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THE METABOLISM AND TOXICITY OF PHENOTHIAZINE

by Stephen Charles Mitchell B.Sc., Ph.D.

A thesis submitted to the Faculty of Science of the
Open University for the degree of Batchelor of Philosophy

Department of Chemistry

November 1985

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ABSTRACT

Phenothiazine, first prepared in 1883 during research into aniline dyestuffs, is the parent molecule of a multitude of drugs which have found varied and extensive use in clinical practice. The compound itself possesses insecticidal, antibacterial, antifungal and anthelmintic properties. Its major use has been as a vermifuge which enabled the provision of many tons of infection-free food and sheep intestine (catgut) which were desperately required during World War II. For this reason alone phenothiazine deserves recognition alongside penicillin and DDT for its remarkable effect on mankind.

To be effective as an anthelmintic the compound has to be given in large doses and because of its high lipid solubility becomes widely distributed around the body. Here it undergoes enzymatically catalysed chemical alterations, mainly oxidations of the carbon and sulphur atoms and conjugation reactions with glucuronic and sulphuric acids.

The therapeutic actions of phenothiazine and the production of its unwanted toxic effects (haemolytic, neuromuscular, photosensitization) presumably arise from common underlying mechanisms, the toxic effects perhaps being aggravated by an environmental or genetic predisposition.

Three basic ways in which the phenothiazine molecule interacts with the cellular components of tissues to produce these observed effects have become apparent from evaluating the available literature. These are the non-specific macromolecular disruptions that can occur to lipid or protein molecules in membranes and other locations, the formation of metabolite redox systems to permit energy transfer to disrupt cellular components and enzyme systems and the formation of allergens with all their immunological sequelae. It is probable that these three mechanisms explain most, if not all, of the actions of phenothiazine.

Statement

Part of the material included in this thesis concerned with the mammalian metabolism of phenothiazine has been previously published in review form.

S.C.Mitchell (1982) Drug Metabolism Reviews 13, 319-343.

List of Contents

	<u>Page No</u>
1. <u>INTRODUCTION</u>	1
A. HISTORICAL	1
B. PHYSICAL AND CHEMICAL PROPERTIES	3
C. USES AND APPLICATIONS	8
1) Insecticidal activity	8
2) Antibacterial activity	10
3) Anthelmintic activity	11
2. <u>METABOLISM AND DISPOSITION</u>	14
A. METABOLISM	14
1) Oxidation	14
2) Conjugation	16
3) Ring degradation	17
4) Biotransformation of phenothiazine metabolites	17
5) Conclusions	20
B. ELIMINATION ROUTES	25
1) Urine	27
2) Faeces	27
3) Bile	28
4) Milk	29
5) Blood	29
6) Other routes	31
7) Conclusions	32
C. PHARMACOKINETICS	32
3. <u>ADVERSE REACTIONS</u>	41
A. INTRODUCTION	41
B. EFFECTS ON BLOOD ELEMENTS	42
C. NEUROMUSCULAR PROBLEMS	45
D. PHOTSENSITIZATION	45
E. OTHER REACTIONS	46

4. <u>MODE OF ACTION</u>	48
A. CONTRIBUTING FACTORS	48
1) Impurities	48
2) Physical effects	50
3) Nutritional factors	50
B. MECHANISM OF ANTHELMINTIC ACTION	51
C. INHIBITION OF ENZYME ACTIVITY	55
1) Sulphydryl interaction	55
2) Redox systems	58
3) Other mechanisms	60
D. PHENOTHIAZINE-INDUCED ADVERSE REACTIONS	61
1) Haemolytic anaemia	61
(i) Direct action	62
(ii) Indirect action	63
(iii) Genetic predisposition	65
2) Neuromuscular problems	67
3) Photosensitization	70
(i) Phototoxicity	70
(ii) Photoallergy	74
5. <u>CONCLUDING REMARKS</u>	77
6. <u>REFERENCES</u>	79

List of TablesPage No

Table 1	Spectral characteristics of phenothiazine	5
Table 2	Recommended dose rates for phenothiazine treatment of livestock	13
Table 3	Metabolism of phenothiazine metabolites	19
Table 4	Urinary metabolites of orally administered phenothiazine	23
Table 5	Relative importance of C-oxidation on the urinary metabolites of various species	24
Table 6	Urinary excretion (24 hour) of phenothiazine and metabolites	26
Table 7	Metabolites present in the blood of various species after the oral administration of phenothiazine	30
Table 8	Urinary excretion rates of phenothiazine in the horse and sheep	35
Table 9	Incidence of haemolytic anaemia in subjects undergoing phenothiazine treatment	44
Table 10	Inhibitory effects of phenothiazine and its metabolites on enzyme systems	56
Table 11	Reduction potentials of thionol at various pH values	59
Table 12	Ultraviolet absorption maxima of phenothiazine and its derivatives	73

List of SchemesPage No

Scheme 1	The mammalian metabolism of phenothiazine	15
Scheme 2	Proposed scheme of phenothiazine oxidation	21
Scheme 3	Redox systems present in phenothiazine metabolites and their possible oxidative interaction with tissue components	54
Scheme 4	Drug-induced photosensitivity	71
Scheme 5	Polymerization of phenothiazine	76

List of Figures

Page No

Fig. 1	Phenothiazine polypeptide conjugates in calf urine	18
Fig. 2	Cumulative urinary excretion of orally administered phenothiazine	34
Fig. 3	Blood levels of phenothiazine and metabolites (total) after oral administration	36
Fig. 4	Semilogarithmic plot of blood radioactivity levels in the guinea pig after oral administration	38
Fig. 5	Biliary secretion of orally administered phenothiazine	39
Fig. 6	Cumulative secretion of orally administered phenothiazine in sheep milk	40
Fig. 7	Structural similarity of phenothiazine and dopamine	68

1. INTRODUCTION

A. HISTORICAL

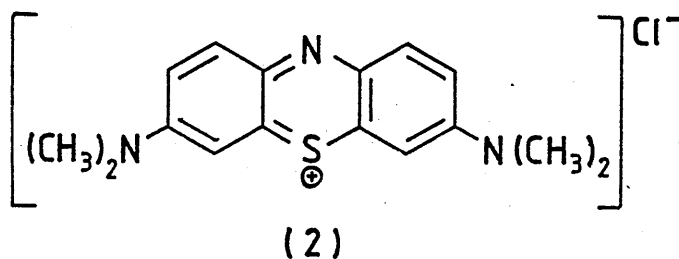
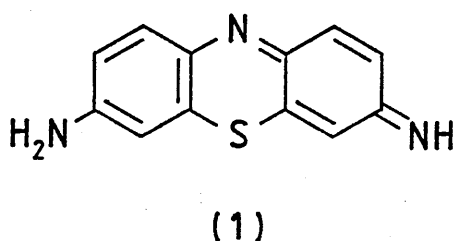
The synthesis of a mauve stain (aniline purple, mauve, mauveine, Tyrian purple) in 1856 by William Perkin and its subsequent production on a technical scale marked the beginning of the 'aniline dye' industry of the latter half of the nineteenth century¹. The value of 'mauve' was first realised in France in 1859, the English and Scottish calico printers not showing any interest until it appeared in French patterns, although some of them had undertaken trials to print cloth with that colour. Contamination of aniline samples with toluidine (methyl aniline) led to the fortuitous discovery, two years later, of aniline red (magenta, rosaniline, solvent red 41, basic violet 14, C.I. 42510). This compound was manufactured by Verguin of Lyons and introduced into commerce under the name of fuchsine.

These early experiments were foreshadowed by those of Runge (1830's) on the oxidation of aniline when he noted various colourations and the formation of a black precipitate which was eventually introduced in 1863 as the valuable pigment Aniline Black. After this initial impetus an extensive search was made for other organic bases capable of being applied to textile fibres in a similar fashion but few were found of any value and none of the importance of aniline.

Perkins 'mauve' was a short lived success. Many other colouring agents had emerged which were not as fast as 'mauve' but were more brilliant. Amongst the colouring matters produced from the products of coal tar were the numerous derivatives of triphenylmethane, including aniline blue (acid blue 22, C.I. 42755), aniline violet

(crystal violet, gentian violet, basic violet 3, C.I. 42555) and aniline green (malachite green, basic green 4, C.I. 42000) discovered by A.W.Hoffmann and methyl violet (basic violet 1, C.I. 42535) synthesized by Charles Lauth in 1861, although the latter compound was not prepared on a large scale until 1867 ².

In 1876, in a note entitled 'Sur une nouvelle classe de matières colorantes' and read by M.Wurtz at the French Academy of Sciences, Lauth's experiments were described in which he heated *p*-phenylenediamine with sulphur and treated the hydrochloric acid solution of the reaction product with ferric chloride to obtain a purple dye (Lauth's violet, thionine, C.I. 52000) (1) ³. In the same year Heinrich Caro carried out a similar reaction with *p*-aminodimethylaniline and obtained a blue dye known as methylene blue (basic blue 9, C.I. 52015) (2) ^{4,5}.

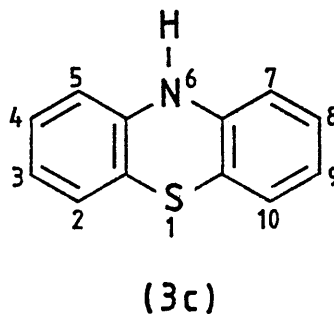
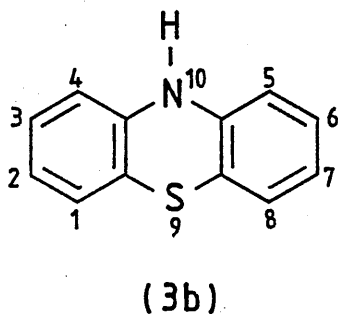
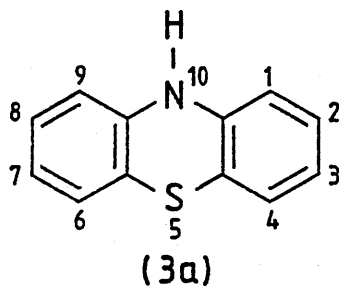


Interested in this new type of artificial dye and suspecting the existence of a then unknown thionated ring system as the nucleus of these substances, Heinrich August Bernthsen set about systematically investigating their composition. During the course of his research he heated diphenylamine with sulphur at 250-260°C and obtained the previously unreported compound, phenothiazine, in 40% yield ⁶. He later proved its structure by a series of elegant studies and showed

that Lauths's violet and methylene blue contained this nucleus ⁷. It was thus during the course of investigations into the structure of two popular artificial dyes that the synthesis and isolation of phenothiazine were first reported in the literature by Bernthsen in 1883 ⁶.

B. PHYSICAL AND CHEMICAL PROPERTIES

Phenothiazine (3), $C_{12}H_9NS$, mol. wt 199.26, was first called thiodiphenylamine by Bernthsen because of its route of synthesis. It has also been referred to in the literature as 2,3;5,6-dibenzo-1,4-thiazine, dibenzoparathiazine, dibenzothiazine, 2-diphenylene-sulpho-imide and phenthiazine ^{6,8}. The numbering system generally applied to the molecule and agreed by the IUPAC Commission is shown below (3a), although other systems have been used (3b, 3c) ⁹.



It crystallises from benzene, toluene or butanol to give yellow leaflets or from alcohol in the form of colourless diamond-shaped plates which become green-brown on exposure to air. The crystals belong to the orthorhombic holohedral class and contain four molecules per unit cell ^{10,11}. The melting point of phenothiazine quoted in the literature varies between 180-186°C depending upon the purity of the sample and the solvent of crystallisation, the generally accepted value for the pure compound from toluene being 185.1°C. The less pure commercial substance has a melting range from 170-175°C ^{12,13}. A non-toxic green oxidation product is present in the commercial preparation to about 1% by weight and can be removed by virtue of its insolubility in diethyl ether. The chemical nature of this dark green contaminant is thought to be a dimer of phenothiazine together with longer polymers ¹²⁻¹⁵.

Sublimation of pure phenothiazine occurs under reduced pressure (1 mm Hg) at 130°C and it is volatile with steam. Boiling points are quoted as 371°C at 760 mm Hg and at 290°C for 40 mm Hg ¹⁶. Density is given as 1.35 ¹¹. The compound possesses a faint but bitter taste, being virtually insoluble in water and carbon tetrachloride. It is only slightly soluble in chloroform, ethanol, petroleum ether (naphtha, b.p. 35-80°C), ligroin (solvent naphtha, b.p. 80-130°C) and mineral oils but dissolves freely in hot acetic acid, benzene, diethyl ether, dimethyl sulphoxide and most other organic solvents. The spectral characteristics of phenothiazine are summarised in Table 1.

Phenothiazine is prepared on a commercial scale by fusing diphenylamine with sulphur, a 30% (by weight) excess of diphenylamine usually being employed. The addition of 0.1 to 1.0% by weight of catalysts such as iodine, aluminium chloride or other electrophilic types reduces the reaction temperature required and increases the yield to almost theoretical ¹⁷⁻¹⁹. The use of a carbon dioxide

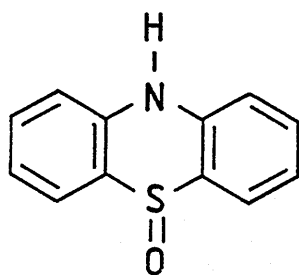
Table 1. Spectral characteristics of phenothiazine.

<u>U.V. absorption maxima</u>		<u>I.R. absorption bands</u>		<u>Mass spectral ions, m/e (% abundance)</u>	
max (nm)	log E	strong.	(KBr disc)		
		(cm ⁻¹)		molecular ion (M ⁺)	other diagnostic ions
<u>methanol</u>					
318	3.6	1476		199 (100)	200 (13)
254	4.5	1445			198 (17)
<u>ethanol</u>					
317	2.4	750			167 (45)
254	3.3	735			166 (17)
<u>acetonitrile</u>					
320	3.7				99.5 (11)
254	4.7				82 (13)
					69 (14)

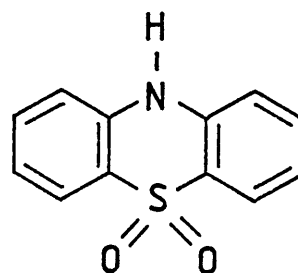
atmosphere has also been shown to improve the process by giving a purer product ²⁰. Final purification is usually afforded by distillation or sublimation followed by washing with carbon tetrachloride ^{13,21-23}. Other routes of synthesis of phenothiazine exist, such as the ring closure of diaminodiphenylsulphide, the thionation of diphenylamine with sodium thiosulphate or arsenic pentasulphide, or from monoarylamines, but these routes are unsatisfactory and usually have very low yields ^{7,16}.

The chemical reactions that phenothiazine can undergo have all been well documented and are presented in detail in several extensive reviews and standard chemistry texts ^{8,16,24-28}. These reactions can be summarised as;

- 1). Aromatic substitution. This is complicated because of side reactions but includes nitration which is comparatively easy, acylation which is somewhat more difficult owing to the competing reaction of the nitrogen centre and halogenation which is usually accomplished indirectly through the sulfoxide as direct halogenation may lead to the formation of perhalides.
- 2). Substitution on the nitrogen atom. The secondary amine group is readily alkylated, acylated and arylated and gives rise to many N-substituted products.
- 3). Oxidation. This can occur at the sulphur atom as well as the carbon atoms of the molecule to give stable and crystallisable compounds. Phenothiazine amine oxides have been reported but their identity is not absolutely proven. The reaction of phenothiazine with hydrogen peroxide or halogenated peracids gives rise to the sulfoxide, phenothiazine-5-oxide (4). The nitrogen atom has to be protected if the sulphone, phenothiazine-5,5-dioxide (5), is required by potassium permanganate oxidation.

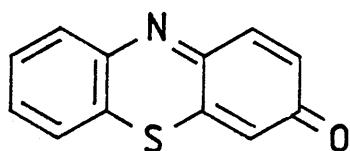


(4)

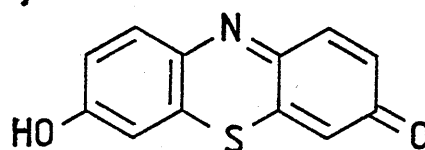


(5)

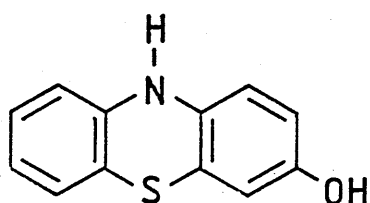
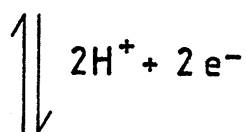
Treatment with FeCl_3 will produce phenothiazone, phenothiaz-3-one (6), and further oxidation of the phenothiazone with H_2SO_4 gives thionol, 7-hydroxyphenothiaz-3-one (7). Both of the latter derivatives are coloured because they possess a para-quinonoid type structure and can be reduced to their colourless hydroxy derivatives (8,9) with $\text{Zn}/\text{NH}_4\text{OH}$.



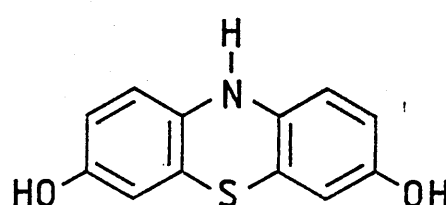
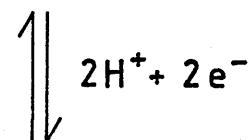
(6)



(7)



(8)



(9)

4). Desulphuration. This can be carried out with a variety of reagents including hydroiodic acid or powdered copper and is useful in the determination of phenothiazine structures.

5). Metalation. With butyl lithium this occurs in the position adjacent to the heterocyclic atom (N) of the ring.

6). Addition to activated double bond. Phenothiazine undergoes addition to vinyl cyanide ($\text{CH}_2=\text{CHCN}$) in the presence of a basic catalyst.

We shall only pursue those chemical reactions of phenothiazine in so far as they relate to the metabolism and likely mode of biological action of the compound.

C. USES AND APPLICATIONS

1). Insecticidal activity.

For some 50 years after its synthesis phenothiazine remained a chemical curiosity. It was an interesting result of systematic chemical research and provided the nucleus for the development of other artificial dyes and biologically active compounds. Indeed, it was and still is the parent compound of a multitude of drugs which have found varied and extensive use in clinical practice. Methylene blue was investigated as an analgesic in 1890²⁹ and was used as the first synthetic antimalarial agent³⁰. In the 1950's the discovery of chlorpromazine and the subsequent renewed interest in N-substituted phenothiazines provided a host of new pharmacologically active compounds. By the early 1970's over 3,000 phenothiazine derivatives had been synthesized and at least one hundred of them have been in clinical use as tranquillisers, antihistamines, antiemetics, sedatives, analgesics and agents for the treatment of Parkinsonism³¹.

However, it was not until 1934 that phenothiazine itself was shown to possess any useful biological properties. In an early report by F.L.Campbell and coworkers ³² sixty-eight compounds containing sulphur were compared to nicotine for their toxicity to culicine mosquito larvae. Twenty-four of these compounds were found to be equal to or exceed nicotine in toxicity and phenothiazine was shown to be even more toxic than rotenone, being effective at concentrations as low as 1 ppm. Shortly afterwards L.E.Smith and colleagues ³³ demonstrated its high toxicity to newly hatched codling moth (apple maggot) larvae. This led to extensive orchard trials and as a result it was frequently used as a fruit spray instead of lead arsenate to control this pest. It was further shown not to affect the quality of or to leave a toxic residue on the fruit and was harmless to the foliage. In addition, it was noticed that the sprayed fruits did not become infected or decay as rapidly as unsprayed produce when stored and a slight fungicidal activity was attributed to phenothiazone (6). This is a carbon oxidation product of phenothiazine formed under suitable weathering conditions, its production being accelerated in the mixtures of phenothiazine, bentonite and lime which were used to facilitate the ease of spraying this insoluble compound ^{34,35}.

Further trials of the insecticidal properties of phenothiazine showed that it possessed variable potency to many species. As well as being lethal by contact to the newly hatched codling moth and mosquito larvae it was also toxic to the Mexican bean beetle, European corn borer, lima bean pod borer, grape berry moth, tomato pinworm and screw worms ^{16,36}. It was shown to act as a stomach poison for the silkworm and the tent caterpillar but had little effect against the cabbage worm, grasshopper, corn ear worm, Japanese beetle, tomato fruit worm, plum curculio, tobacco hookworm or the boll weevil ^{16,36}. Phenothiazine

in fruit sprays has also been shown to favour the development of spider mite populations ³⁷. The American cockroach was not affected when fed phenothiazine but succumbed when it was applied to the cuticle ³⁸. In addition, honey bees could ingest as much as 570 ug without any apparent toxic effects ^{27,39}.

2). Antibacterial activity.

It was noticed that the urine from rats receiving oral phenothiazine turned red on exposure to air but did not develop the usual offensive odour on standing. Subsequent examination revealed the lack of gross evidence of bacterial growth in the urine after standing exposed for several weeks ⁴⁰. Preliminary tests showed that a retardation in the growth of Escherichia coli added to the urine of rats, rabbits and a human previously fed with phenothiazine occurred after incubation for 24 hours when compared to additions to normal urine from untreated animals, suggesting an antiseptic action of phenothiazine or its metabolites. Thionol (7), a urinary metabolite of phenothiazine, was also shown to possess a bacteriocidal action against Staphylococcus aureus in vitro ⁴¹.

Further investigations showed that the oral administration of phenothiazine brought about some improvement of experimentally induced cystitis (E.coli) in male rabbits. Tests in humans with both chronic and acute urinary tract infections showed that 15 patients (5 chronic, 10 acute) were clinically cured and that only 8 out the 49 failed to secure relief from symptoms of the urinary tract inflammation following phenothiazine therapy ⁴².

Investigations into the nature of the bacteriocidal action of phenothiazine indicated that it was not a property of the parent compound but of its oxidation product, thionol ⁴¹. Interestingly, phenothiazine itself has been shown to inhibit the growth of tubercle

bacilli in vitro in high dilution (1 ppm), the bacteriostatic effect being diminished in the presence of serum but still significant, whereas the oxidised forms of phenothiazine only showed a moderate degree of inhibition (10 ppm) ⁴³.

3). Anthelmintic activity.

In 1938 phenothiazine was reported to be effective in preventing the development of horn fly larvae in the faeces of cattle previously treated with oral doses of this compound ⁴⁴⁻⁴⁶. About the same time a trial was carried out by E.L.Taylor and K.M.Sanderson on pigs infected with ascaris at the veterinary laboratory of the Ministry of Agriculture, but no definite conclusions were arrived at ⁴⁷. However, P.D.Harwood and colleagues at the Bureau of Animal Industry managed to demonstrate a rather variable but nevertheless marked action of the compound in removing ascarids and nodular worms from swine ⁴⁸. In 1939 the compound was found by several workers in Australia and Canada to be effective against strongyloid worms in sheep and this was afterwards confirmed by Taylor and Sanderson for the A.R.C. in England ^{47,49}. After these initial investigations many more demonstrations of the anthelmintic activity of phenothiazine were presented and these have been subsequently more than adequately reviewed in the literature ⁵⁰⁻⁵².

For a time phenothiazine was viewed as a panacea for parasitic infections. The use of the compound to control livestock parasites, with its powerful indirect effect on human health, overshadowed its other values. In the United States the production of phenothiazine rose from 408 kg in 1939 to 1,260,300 kg in 1943, largely for anthelmintic consumption. In 1946 the U.S. production was estimated at 1,788,200 kg ^{37,38}. It has been cited that between December 1938

and January 1944 approximately 900 articles had appeared on the use of phenothiazine as an anthelmintic ⁴⁸. Folse, in his comprehensive review, accounted for about 400 of these, perhaps indicating the avid interest of industry and that many of these articles were internal publications ⁵³. The same author listed over 1,500 publications concerning phenothiazine which had appeared in the world literature between 1934 and 1958 and demonstrated a rapid increase during the early 1940's. It was during this period that the use of phenothiazine against sheep parasites was estimated as preventing an annual loss of one million dollars in the State of Kentucky alone ³⁹.

The compound was used extensively in ruminants throughout the world for some 20 years after its introduction and, although still used on a small scale as a cheaper form of therapy and as a prophylactic in the control of manure-breeding flies, it effectively disappeared from the farming scene in the mid-1960's being superseded by more efficient and safer broad spectrum anthelmintics, such as thiabendazole. However, during the period of its widespread popularity many millions of animals were successfully treated with phenothiazine at dosage rates that may be considered large when compared with modern anthelmintics ⁵² (Table 2). It was this availability of countless numbers of animals coupled with the wide financial and scientific interest in the drug that provided the opportunity and the incentive to investigate the compound in detail and thus give sufficient information to construct an overall picture of the mammalian metabolism of this anthelmintic and to permit an insight into the possible mechanisms of its toxicity.

Table 2. Recommended dose rates for phenothiazine treatment of livestock.

	Single dose	Repeated dose
Horse	10 to 30g (3g per 50kg body wt)	2 to 5g (foals 1 to 2g)
Cattle	20 to 60g (10g per 50kg body wt)	0.5 to 5g (0.5g per 50kg body wt)
Sheep & Goats	5 to 40g	0.25 to 0.5g
Poultry	0.25 to 1g	- - - - -

Phenothiazine is no longer recommended for pigs, dogs or cats.

Young foals, calves under 3 months, lambs under 1 month and heavily pregnant animals should not be dosed. Doses for sick and debilitated animals should be calculated with caution.

Data taken from reference 52.

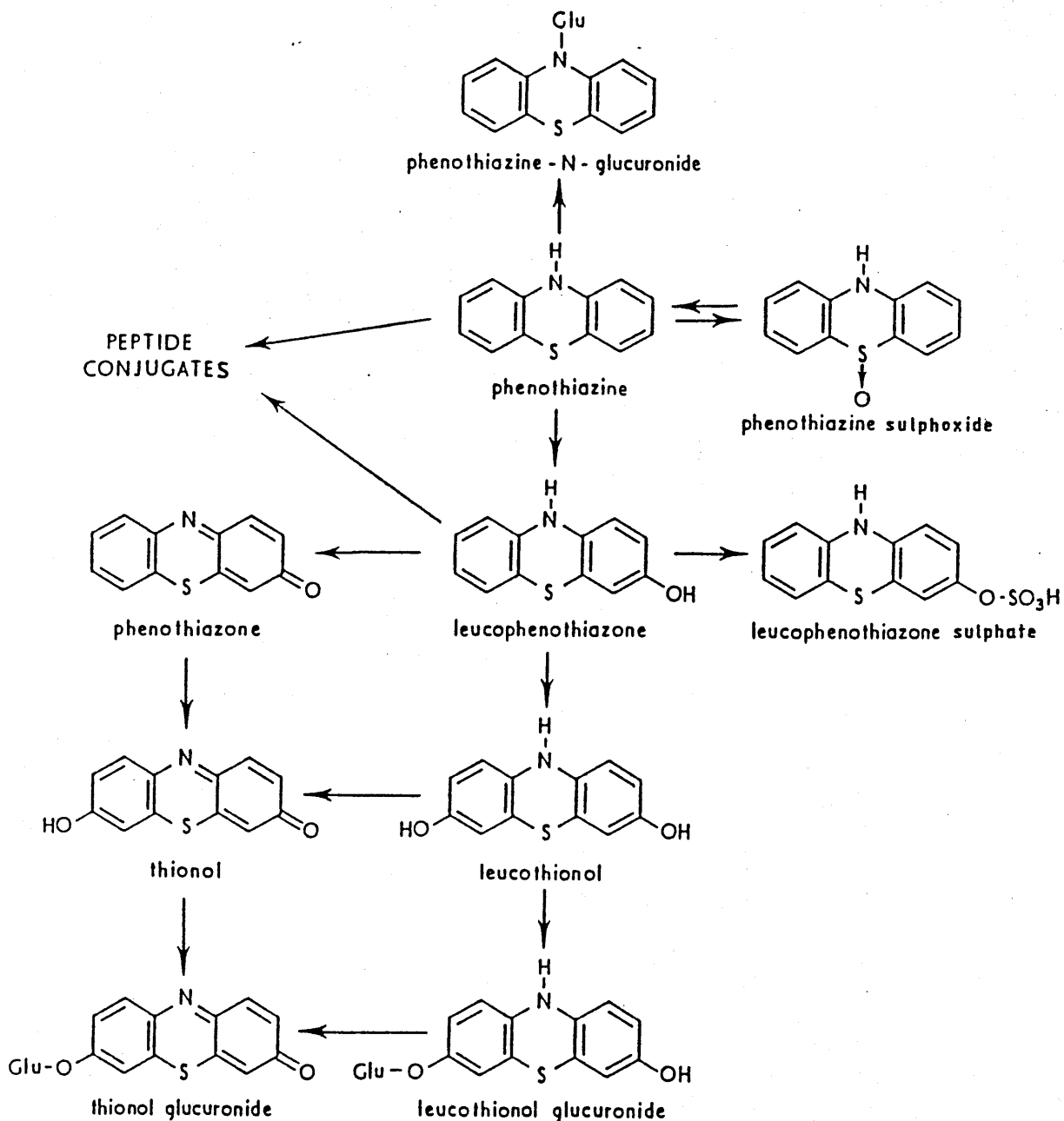
2. METABOLISM AND DISPOSITION

A. METABOLISM

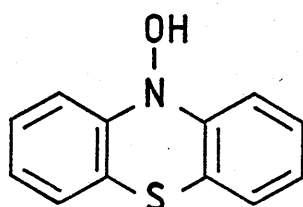
Phenothiazine (3), like many other foreign compounds, undergoes chemical modification during its passage through the mammalian body before being expelled into the environment. Synthetic (conjugation) reactions with endogenous substrates, resulting in the formation of more polar and water-soluble condensation products, can occur either directly with a drug or after a reactive site has been introduced by modification. Such alterations can take the form of oxidation, reduction or hydrolysis. Phenothiazine only exhibits oxidation.

1). Oxidation.

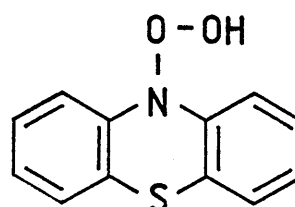
As illustrated in Scheme 1, C-oxidation occurs para to the nitrogen atom in the heterocyclic structure to give leucophenothiazone (3-hydroxyphenothiazine)(8) and leucothionol (3,7-dihydroxyphenothiazine) (9). These phenolic derivatives may be further oxidised, especially on exposure to the atmosphere, to yield the corresponding coloured quinoid structures, phenothiazone (6) and thionol (7) respectively. S-Oxidation gives phenothiazine sulphoxide (4) which is excreted unchanged and there is no evidence that it is metabolised further to the sulphone (5) or excreted in a conjugated form although it may undergo further interconversions in vivo. The formation of amine oxides (10,11) has been reported to occur during incubation of guinea pig liver microsomes with phenothiazine ⁵⁴ but in vivo studies have failed to show any N-oxidation products ⁵⁵ and recent reports suggest that those previously identified were in fact C-oxidation compounds ^{56,57}.



Scheme 1. The mammalian metabolism of phenothiazine.



(10)



(11)

2). Conjugation.

Leuco-phenothiazine is conjugated with sulphuric acid to give the colourless leuco-phenothiazine sulphate, whilst leucothionol/thionol conjugates with glucuronic acid to form an ether glucuronide which may exist as either the colourless leucothionol conjugate or thionol glucuronide containing the coloured phenothiazine moiety (Scheme 1). It is probable that the two structures are easily interconvertible and that the glucuronide is either initially excreted in the coloured quinoid form or that it is rapidly oxidised on contact with the air after excretion⁵⁸. Direct conjugation of phenothiazine without previous oxidation is possible and has been often reported in the literature as a 'water-soluble acid-labile conjugate of phenothiazine' recently been given the assignment of an N-glucuronide⁵⁵.

A polypeptide conjugate of phenothiazine containing at least six amino acids including arginine, glutamic acid, phenylalanine and tyrosine has also been observed in the urine of calves up to six weeks old^{59,60}. More recent investigations have shown that a polypeptide conjugate fraction, accounting for about 3% of the administered dose,

could be isolated from the urine of three day old male Friesian calves after treatment with phenothiazine. This fraction consisted of four different polypeptide conjugates containing two, three, five and six amino acids respectively, attached to the nitrogen atom of phenothiazine or leucophenothiazone by means of the carboxyl terminal of the peptide chain (Fig. 1) ⁶¹.

3). Ring degradation.

Although it has been reported that about 10% of a dose of (³⁵S)-phenothiazine given to dogs was excreted in the urine as labelled inorganic sulphate which could be precipitated with barium chloride ⁶², it has recently been found that both inorganic sulphate and leucophenothiazone sulphate were precipitated by barium chloride from the urine of guinea pigs dosed with (³⁵S)-phenothiazine. Subsequent chromatography showed that the radiolabel was still associated with the phenothiazine nucleus. In addition, animals fed (¹⁴C)-phenothiazine did not expire any radioactive carbon dioxide indicating that the ring structure was not degraded during its passage through the guinea pig body ⁵⁵.

4). Biotransformation of phenothiazine metabolites.

Experiments with sheep, rabbits and guinea pigs have shown that orally administered phenothiazine sulfoxide is eliminated in the urine as both phenothiazine and phenothiazone but not in the unchanged form, the sulfoxide itself being detectable only in the faeces. Dosing with phenothiazone or thionol only leads to the excretion of the compounds themselves, either free or conjugated, and there is no evidence that the C-oxidation metabolites undergo in vivo conversion to phenothiazine or its sulfoxide (Table 3) ^{55,63-65}.

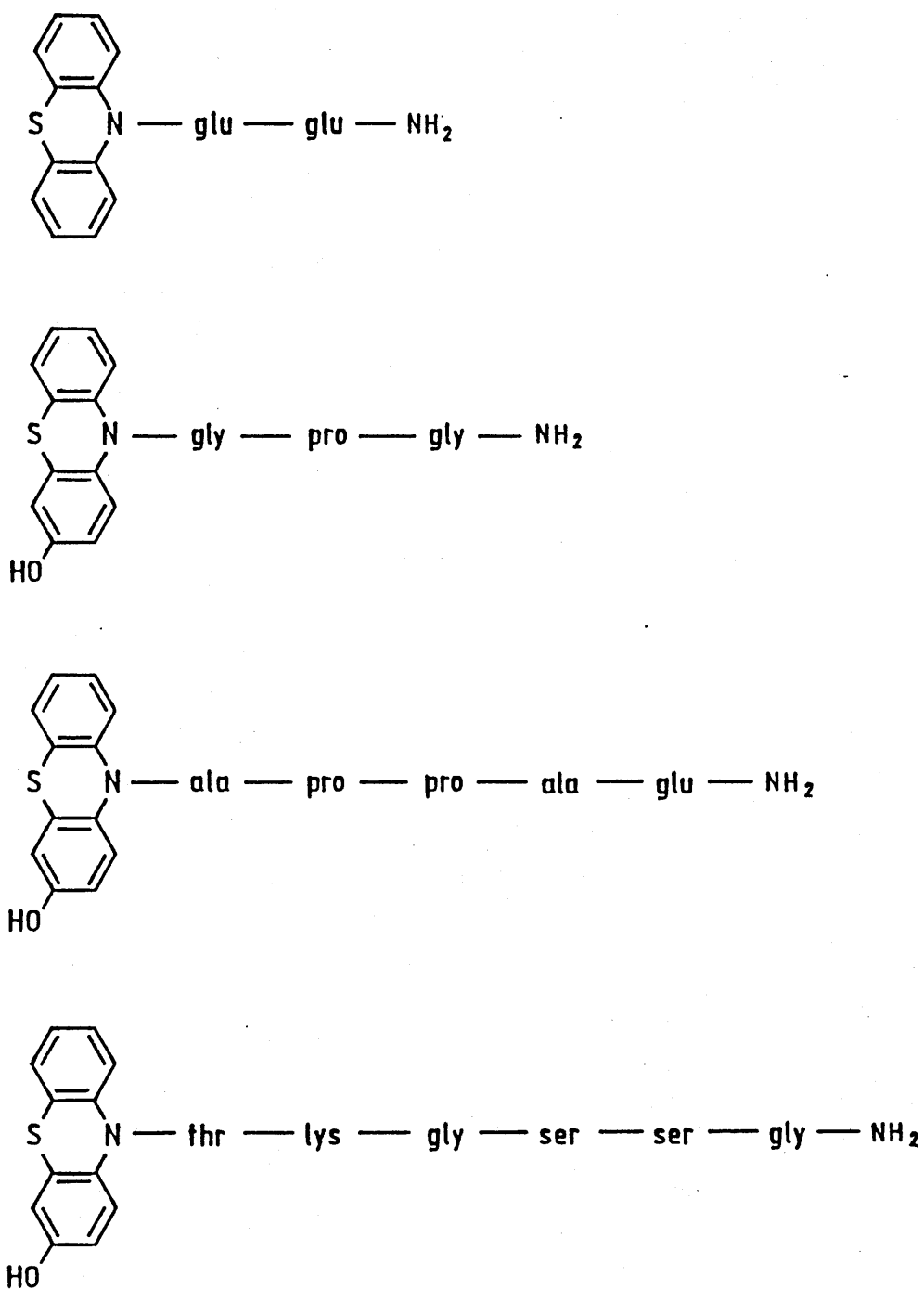


Fig. 1. Phenothiazine polypeptide conjugates in calf urine.

Table 3. Metabolism of phenothiazine metabolites.

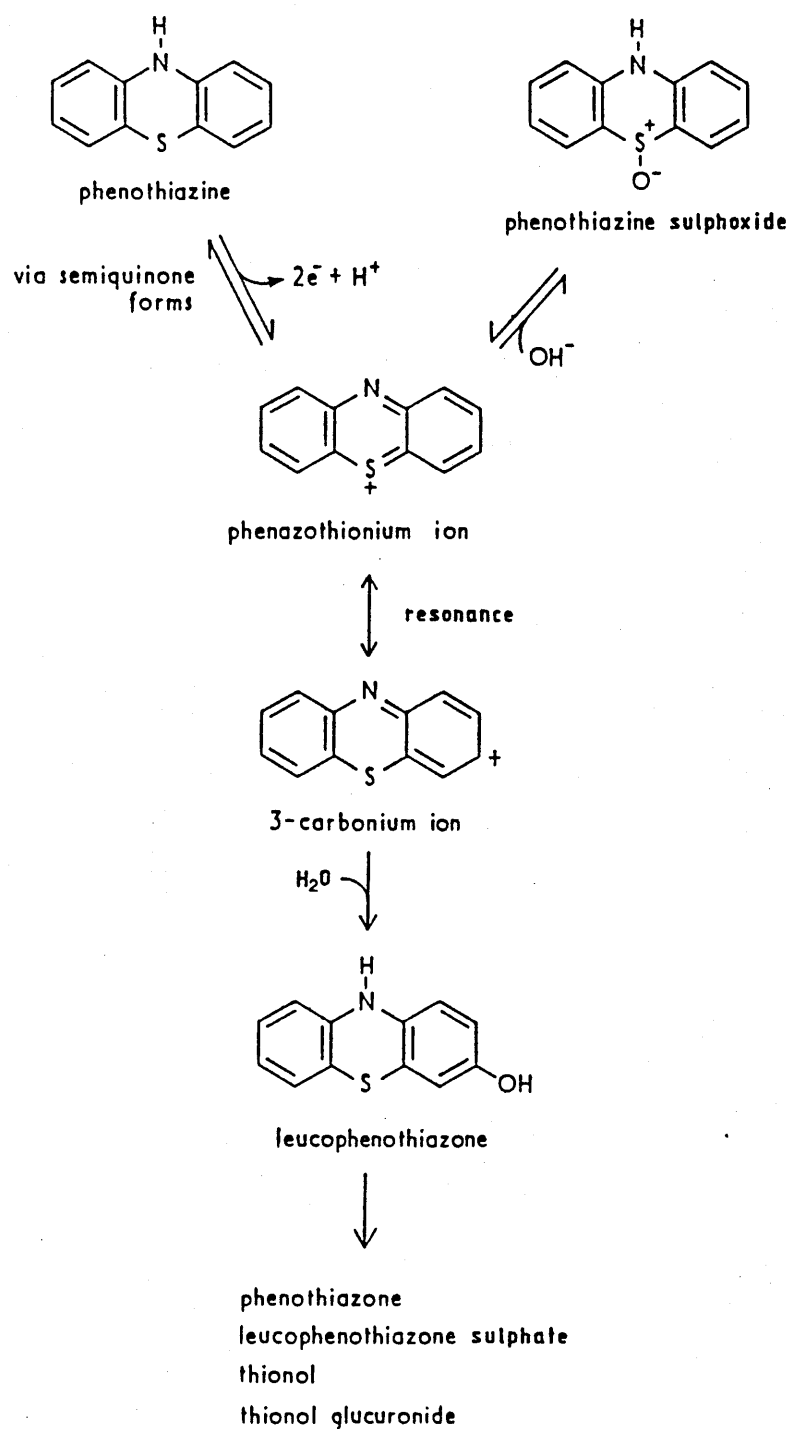
Oral dose	Species	Urinary metabolites			
		phenothiazine	phenothiazone	thionol	leucophenothiazone sulphate
phenothiazine sulphoxide	sheep		+		
	rabbit	+	+		
	guinea pig	+	+		+
phenothiazone	sheep		+		
	rabbit		+		+
	guinea pig		+	+	
thionol	rabbit			+	
	guinea pig			+	

+ indicates metabolite detected in urine. Data from references 55, 63-65.

Phenothiazine and phenothiazone are known to be interconvertible in strong acid solution ⁶⁶ and a route has been proposed for the oxidation of phenothiazine (Scheme 2) ^{15,67}. This sequence proceeds via electron and proton removal to give the phenazothionium ion, the most stable resonance form of which is shown. Another resonance form is the 3-carbonium ion and attack at C-3 results in the formation of leucophenothiazone as a stable end product. The subsequent production of leucophenothiazone sulphate is essentially an irreversible process. Phenothiazine sulfoxide, however, has been shown to be in equilibrium with the phenazothionium ion ^{15,67}. This postulated mechanism appears to be in agreement with experimental observation and places the formation of the sulfoxide on a spur line in the biotransformation route and not in the direct role as an active intermediate in metabolism. It is possible that the transformation of the phenazothionium ion to leucophenothiazone is rate-limiting in which case the competing sulfoxide formation, which is reversible, acts as a temporary reservoir for phenazothionium ions.

5). Conclusions.

Very little in vitro work has been carried out on the metabolism of phenothiazine itself mainly because of its low water solubility. However, incubations of phenothiazine with guinea pig liver homogenates and microsomes derived from hamster liver have demonstrated the formation of phenothiazine sulfoxide together with the carbon oxidation products ^{63,68}. It is presumed that phenothiazine, by analogy with investigations using the water soluble N-substituted derivatives of phenothiazine, notably chlorpromazine ^{69,70}, is oxidised by a microsomal cytochrome P-450-containing monooxygenase system mainly within the liver, although these oxidation reactions may well occur at other sites such as within the mucosa of the small intestine. Indeed, the oral



Scheme 2. Proposed scheme of phenothiazine oxidation.
 Redrawn and simplified after Refs. 15 & 67.

pretreatment of rats with phenothiazine in the diet for several days has been shown to bring about an induction of cytochrome P-450 mediated microsomal hydroxylations in the liver, kidney and intestine ⁷¹, ⁷². The conjugation of phenothiazine and its hydroxylated metabolites also presumably takes place in the liver, although this too may occur at other sites, the gut wall being a prime candidate for sulphation.

The basic pattern of metabolism, as summarised in Scheme 1, is the same for all mammals studied with the exception of the phenothiazone-peptide conjugates from young calves although such metabolites may exist in the urine of other rapidly developing neonates of many species as yet uninvestigated ⁶¹. From the accumulated results of urinary metabolites a comparison of species can be made, though it must be remembered that very few quantitative results are available and that failure to mention the presence of a metabolite may simply indicate that it was not sought (Table 4). For example, Benham ⁸⁵ has produced an excellent paper in which he described the then-novel metabolite, thionol glucuronide, present in rabbit urine but made no mention of the N-glucuronide or unconjugated metabolites detected by other workers (Table 4).

All species studied excrete the majority of their urinary products in conjugated form (> 80%), predominantly the N-glucuronide and leucophenothiazone sulphate. A basic difference appears to lie in the percentage of the drug which undergoes C-oxidation (Table 5) rather than whether or not conjugation occurs with glucuronic or sulphuric acid as both of these synthetic reactions appear to be available in nearly all species (Table 4). Accurate quantitative data are not available for all species, but they tend to fall into two groups, those in which C-oxidation appears to predominate (dog, horse, sheep, cow, rabbit, gerbil, rat and mouse) eventually giving rise to leucophenothiazone sulphate and thionol glucuronide and those in

Table 4. Urinary metabolites of orally administered phenothiazine.

Species	Unconjugated metabolites			Conjugated metabolites		
	phenothiazine	phenothiazine sulphoxide	phenothiazine thionol	phenothiazine glucuronide	leucophenothiazone sulphate	thionol glucuronide
Man	+	+	+	++	++	
Dog		+	+		++	
Horse		+	+		++	
Pig		+	+	++	+	+
Sheep	+	+	+	+	++	
Cow	+	+	+	+	++ ^a	
Rabbit	+		+	++	+	++
Guinea pig	+	+		++	++	
Gerbil			+	++	++	+
Hamster			+	++	+	
Rat		+	+	+	++	++
Mouse		+	+	+	++	+

+ indicates a minor metabolite (up to 20%); ++ indicates a major metabolite (20% and over).
a only present in animals over 4 weeks old, calves up to 6 weeks excrete a polypeptide conjugate (59,60).

References: Man (63,73-75); Dog (64,73); Horse (64,76); Pig (60,64); Sheep (59,60,64,65,77-80); Cow (49,59,60,81-83); Rabbit (63-65,73-75,84,85); Guinea pig (55); Gerbil (58); Hamster (58); Rat (58,74,75); Mouse (58).

Table 5. Relative importance of C-oxidation on the urinary metabolites of various species.

Species	% Dose excreted in 0-24 hour urine	
	Conjugates	Undergoing C-oxidation
Man	92.3	32.6
Guinea pig	83.3	31.4
Gerbil	96.0	70.4
Hamster	97.0	13.4
Rat	93.2	82.0
Mouse	90.0	77.1

Data taken from references 55,58,63.

which it accounts for a third or less of the urinary output (man, pig, guinea pig and hamster). The later group excrete the majority of their dose in the form of phenothiazine-N-glucuronide which may suggest a less efficient microsomal oxidation system for phenothiazine in these species. However, the reverse situation may be more probable in which the apparent poor C-oxidation group may have extensive glucuronyl transferase activity towards this substrate in the gut wall thereby forming relatively large amounts of the N-glucuronide before the compound reaches the liver and thereby effectively removing it as a substrate for subsequent C-oxidation. Further work needs to be undertaken in this area before this problem can be resolved.

In addition to the species mentioned previously, phenothiazine has been administered therapeutically to many other kinds of animals including the bear (polar & brown)⁸⁶, beaver⁸⁷, bison⁸⁸, camel⁸⁹, cat⁹⁰, chicken^{81,91}, deer^{92,93}, elephant⁹⁴, fox⁹⁵, geese⁹⁶, pheasant^{97,98}, pidgeon⁹⁹, sable¹⁰⁰, weasel¹⁰⁰ and yak¹⁰¹ but very little useful metabolic data have been cited. The (³⁵S)-labelled compound has been shown to be taken up by nematode parasites, desulphuration of the heterocyclic structure not being demonstrable¹⁰²⁻¹⁰⁵, and Zukel³⁸ has stated that phenothiazine eventually kills the cockroach (Periplaneta americana) by the accumulation of a leucothionol conjugate in the haemolymph but does not detail its metabolism.

B. ELIMINATION ROUTES

The major routes of elimination of phenothiazine and its derivatives from the mammalian body are via the urine and faeces; even so it takes several days or even weeks for near-total elimination to be achieved. Many workers have found low 24 hour urinary recoveries of administered drug, all values being less than 45% (Table 6).

Table 6. Urinary excretion (24 hour) of phenothiazine and metabolites.

Species	Oral dose rate (mg/kg body wt)	% Dose excreted α
Man	6	25.4
Horse	55-130	9-12
Sheep	220-840	10-20
Cow	220	24.8
Rabbit	1500	25.0
Guinea pig	150	37.1
Gerbil	150	29.9
Hamster	150	44.1
Rat	150	18.2
Mouse	150	44.0

α calculated as percentage administered radioactivity or sum of excreted metabolites.
Data taken from references 55, 58, 63, 76, 78, 79, 85, 107.

In the few quantitative studies concerning faecal excretion some 8 to 44% of the drug was recovered from sheep by this route over five days^{84,106}. Faecal excretion by other species was 24 to 29% in 2.5 days from horses⁷⁶, 23% during the first day from cows¹⁰⁷ and 7.7% in 24 hours (23.9% in 7 days) from guinea pigs⁵⁵. In addition, phenothiazine has been shown to still be present in sheep for 14 to 16 days¹⁰⁸, in calves for 5 to 15 days¹⁰⁹ and in humans for 6 to 9 days^{110,111} after administration was discontinued.

1). Urine.

This is the most important route of excretion for that part of the dose which has actually entered the mammalian body after absorption as opposed to that which is carried through the enteric tract unabsorbed to be voided in the faeces. It is also the route that has been most extensively investigated and in which the greatest number of metabolites have been found. The available published data are summarised in a semi-quantitative fashion in Table 4. The absence of a metabolite in the urine may reflect its true absence or its presence in such small amounts that it has gone undetected or even that it was simply not looked for. Insufficient information is given in the literature to determine which of these is the case.

2). Faeces.

Phenothiazine is the only compound that has been positively identified as being present in the faeces of sheep^{80,84,106}, cows⁸³, horses⁷⁶, rabbits⁸⁴, rats⁶³ and guinea pigs⁵⁵, presumably unabsorbed, although trace amounts of phenothiazine and thionol have been found in rat faeces⁶³ and phenothiazine and phenothiazine sulphoxide in guinea pig faeces⁵⁵.

It is probable that oxidation products also occur in the faeces of the other animals examined but in quantities too small to be readily detected. Phenothiazone and phenothiazine sulphoxide have been observed in the gut of sheep distal to the bile duct and in vivo and in vitro experiments with rumen contents have shown that phenothiazine was not converted into any detectable derivatives except for minute traces of phenothiazone probably arising from air oxidation ⁸⁴, indicating that the compounds entered the enteric tract via the bile ^{59,77,112}. Phenothiazone has also been detected in the gut of cows ⁸¹ and with thionol in the gut of chickens ¹¹³.

3). Bile.

Phenothiazone, both in the free and conjugated forms, has been reported in the bile of sheep together with traces of the sulphoxide but no phenothiazine was detected ^{59,64,77}. These results are unlike those from cows where phenothiazine, phenothiazone and traces of the sulphoxide were present ^{59,60,81}. Minute amounts of phenothiazine, phenothiazone and thionol were found in chicken bile ^{102,113}, phenothiazone and an unidentified glucuronide (presumably phenothiazine-N-glucuronide) in rat bile ⁶³ and phenothiazine-N-glucuronide was present in the bile from guinea pigs together with trace amounts of phenothiazine probably from the breakdown of the conjugate ⁵⁵. Phenothiazine, leucothionol and thionol were identified in the bile from rabbits, dogs and a human subject but the detection methods used did not permit the differentiation between phenothiazine and its N-glucuronide or between the C-oxidation products ⁷³.

Overall biliary secretion seems to account for only a few percent of the administered dose (eg. 2.7% dose in 0 to 24 hour guinea pig bile ⁵⁵) and is not a major route of elimination of the drug.

4). Milk.

A pink discolouration occurring after exposure to air for several hours was noticed with goat milk ^{114,115} and cow milk ^{46,116} collected from animals treated with phenothiazine. Such cow milk gave a mauve colour upon addition of concentrated hydrochloric acid ¹¹⁷, the pink chromogen being identified as a phenothiazone derivative ⁸³. Similar observations have been made with sheep milk where leucophenothiazone and its sulphate conjugate have been identified ^{79,80}.

5). Blood.

The phenothiazine derivatives identified in the bloodstream of the five species that have been studied are shown in Table 7.

Undifferentiated radioactivity has also been found in the blood of hamsters after intraperitoneal dosage of (³⁵S)-phenothiazine ¹¹⁸.

Clare has stated that differences may be observed depending on where the blood is sampled and has shown that in sheep the portal blood contained both phenothiazone and the sulphoxide whereas that in the systemic circulation contained only phenothiazone ⁵⁹.

Collier found that haemolysed erythrocytes from sheep treated with phenothiazine contained no leucophenothiazone sulphate although it was present in the serum ⁸⁰ and it has also been shown that phenothiazine does not penetrate erythrocytes from the plasma ¹¹⁹.

In agreement with this finding the 2.5% or so of the total radioactivity present in the cell fraction of guinea pig blood was shown to be associated with their membranes and not their contents, indicating adsorption to the erythrocyte surface but no passage into the cell ^{55,63}. This presumably reflects the simple partition of phenothiazine between lipid and aqueous phases with the compound being much more soluble in organic material. In addition, when phenothiazine ($2.5 \times 10^{-5}M$) was added to equal volumes of a suspension (1:20) of human erythrocytes

Table 7. Metabolites present in the blood of various species after the oral administration of phenothiazine.

Species	Phenothiazine metabolites			
	phenothiazine	phenothiazine sulphoxide	leucophenothiazine & phenothiazine	leucothionol & thionol
Pig		ab +	ab +	a +
Sheep		abc +	ab +	ac +
Cow	c +	ab +	c +	a +
Guinea pig	a +	a +		a +
Chicken	ab +	b +		b +

+ indicates metabolite detected (a = whole blood; b = plasma; c = serum).
Data taken from references 55,59,60,77,79-81,102,122.

approximately half of the compound was found to be bound to the cells¹²⁰. Phenothiazine and thionol have also been shown to bind to bovine serum albumin (also yeast nucleic acid and calf thymus nucleohistone) but it was found that this binding, although moderately strong, was not extensive with only two binding sites being available per mole of albumin protein (cf. methyl orange which has 22). However, owing to the low aqueous solubility of these metabolites all the potential binding sites may not have been saturated with the compounds¹²¹.

6). Other routes.

Metabolic studies using (¹⁴C)-homocyclic ring labelled phenothiazine in the guinea pig showed that no detectable radioactivity was expired during the three days after dosing indicating that pulmonary excretion was not a significant route of elimination⁵⁵. Similar conclusions were drawn from work with cows¹⁰⁷.

Investigations into the aetiology of photosensitized keratitis (see later) occurring in phenothiazine treated animals led to the discovery of phenothiazine sulphoxide, but no other derivative, in the aqueous humour of calves and occasionally in sheep that were treated with high doses^{59,122}. Nonspecific experiments using (³⁵S)-phenothiazine have shown that radioactivity was present in the uveal tract of hamsters and rabbits but in insignificant amounts when compared with the uptake of N-substituted phenothiazines¹¹⁸. Phenothiazine sulphoxide has also been found in the lacrimal fluid of young calves⁵⁹. Studies with cats, monkeys and humans have shown that this radioactive phenothiazine is selectively taken up by different parts of the brain, particularly the brain stem, and that this specific distribution could not be accounted for solely by the density of vascular tissue present¹²³. It is presumed that the compounds are transported to the lipid-rich neuronal areas of the uveal tract and brain by the bloodstream and then preferentially

enter these areas, gaining access through the blood-brain barrier simply because of their high lipid solubilities.

During the treatment of pregnant goats with phenothiazine it was noticed that after parturition the entire integument of the white kids was stained pink, suggesting excretion of C-oxidation products into the amniotic fluid bathing the foetus in utero¹¹⁵. Blood obtained from the foetus of a pregnant ewe contained no detectable leucophenothiazone indicating that it did not pass across the placenta⁷⁹.

7). Conclusions.

In general, phenothiazine is poorly absorbed from the gastrointestinal tract after oral administration. However, enough material of the large therapeutic dose is absorbed into the bloodstream and distributed throughout the body to cause toxic effects. This wide distribution throughout the tissues has been demonstrated during postmortem examination of phenothiazine-treated cattle in which the C-oxidation products were found in the liver, spleen and mesenteric lymph nodes⁸¹, while after a prolonged exposure to the air the whole carcass took on a red colouration⁴⁹. Radioactive phenothiazine has also been shown to cross the blood-brain barrier¹²³. It is this wide distribution together with its low aqueous solubility - high lipid solubility and protein binding which leads to its prolonged retention in the mammalian body.

C. PHARMACOKINETICS

Disappointingly little pharmacokinetic data can be deduced from the literature because most published results are incomplete, not possessing sufficient detail and lacking specificity in quantitative metabolite determinations. For example, the earlier papers measured

total drug output by oxidation of the parent compound and all metabolites including conjugates to phenothiazone or a bromine derivative of thionol, both of which were highly coloured and easily quantitated by simple colorimetric methods ^{76,79,124}. Other workers only recorded values for the first few hours after dosing or a single urine collection of 0 to 24 hours thus giving only one or two data points and making any pharmacokinetic analysis impossible.

Nevertheless, cumulative urinary excretion curves can be constructed for man, horse and sheep (Fig. 2). These plots indicate that the largest amount of drug is excreted within the first 24 hours after dosing and that the fastest rate of excretion (mg hr^{-1}) occurred in the 0 to 9 hour period in man, horse, sheep and cow ^{63,76,79,81,125}. A more detailed examination of the excretion rates in horse and sheep (Table 8) shows a maximum in the 4.0 to 7.5 hour period followed by a second increase during the 12.0 to 31.5 hour collection periods. This could possibly reflect reabsorption after biliary secretion, a similar pattern being observed during studies with a human volunteer ¹²⁵. However, this phenomenon was not observed for all animals studied ^{76,79} although this may have been due to inappropriate collection periods.

A few investigations have been undertaken where the drug concentration in serial blood samples has been monitored and the results are shown collectively in Fig. 3. The sheep is the animal in which the majority of the reported studies have been performed and it can be seen that values rise to a maximum within the first nine hours after dosing and then slowly decline, still being detectable in some animals after 72 hours. One animal (C) shows a peak value at 24 hours although this anomaly is almost certainly due to insufficient samples being taken over this initial period ⁷⁹. Values for one horse are also available and show a very similar pattern ⁷⁶ (Fig. 3). The quantity of drug present at any one time in the circulatory system only represents

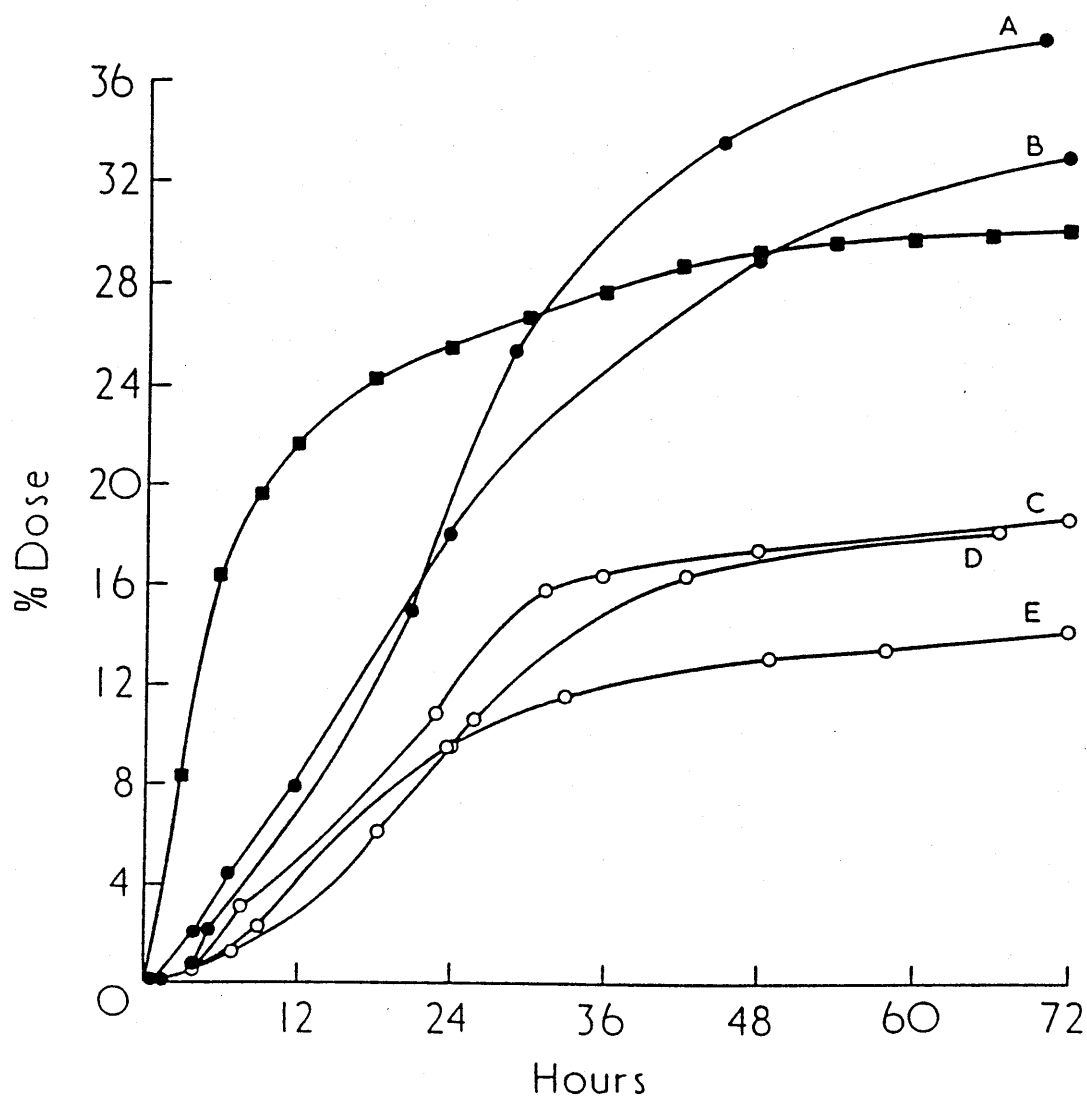


Fig. 2. Cumulative urinary excretion of orally administered phenothiazine.

Each graph line represents one animal.

- Man (dose 423mg; 6mg/kg body wt) measured radioactively.
- Sheep (A- dose 21.9g; 420mg/kg body wt; B- dose 42.5g; 815mg/kg body wt) measured as phenothiazone after oxidation.
- Horse (C- dose 60.0g; 110mg/kg body wt; D- dose 30.0g; 53mg/kg body wt; E- dose 70.0g; 123mg/kg body wt) measured as phenothiazone after oxidation.

Drawn from recalculated data (Refs. 63,76,79).

Table 8. Urinary excretion rates of phenothiazine in the horse and sheep.

<u>A</u>		<u>B</u>		<u>C</u>	
Collection period (h)	Excretion rate (mg h ⁻¹)	Collection period (h)	Excretion rate (mg h ⁻¹)	Collection period (h)	Excretion rate (mg h ⁻¹)
0.0 - 4.0	120	0.0 - 1.25	14	0.0 - 0.5	10
4.0 - 7.5	540	1.25 - 4.0	60	0.5 - 4.0	260
7.5 - 23.0	380	4.0 - 5.0	330	4.0 - 6.5	450
23.0 - 31.5	450	5.0 - 21.0	190	6.5 - 12.0	290
31.5 - 36.0	110	21.0 - 29.0	310	12.0 - 24.0	390
36.0 - 48.0	70	29.0 - 45.0	120	24.0 - 48.0	210
48.0 - 72.0	40	45.0 - 69.0	38	48.0 - 72.0	76
72.0 - 95.0	27	69.0 - 93.0	8	72.0 - 96.0	27
95.0 - 143.0	6			96.0 - 102.0	15

A = Horse, dose 60g (110 mg/kg body wt.); B = Sheep, dose 21.9g (420 mg/kg body wt.); C = Sheep, dose 42.5g (815 mg/kg body wt.).

The total drug excreted was determined as phenothiazone after oxidation.

Recalculated from references 76,79.

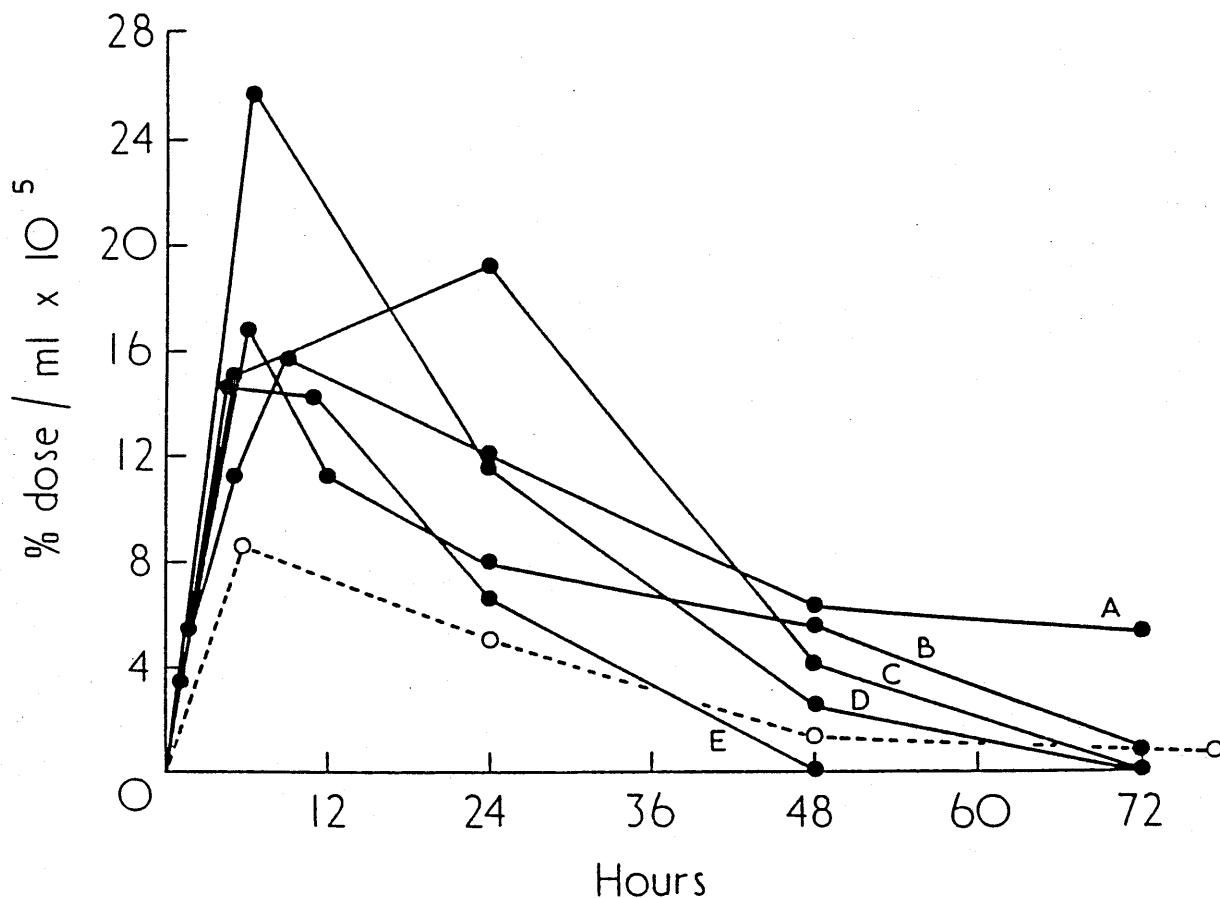


Fig. 3. Blood levels of phenothiazine and metabolites (total)
after oral administration.

Each graph line represents one animal.

- Sheep (A- dose 47.0g; 837mg/kg body wt; B- dose 42.5g; 815mg/kg body wt; C- dose 21.9g; 420mg/kg body wt; D- dose 38.0g; wt not specified - lactating ewe; E- dose 38.0g; wt not specified - lactating ewe).

- Horse (dose 70.0g; 123mg/kg body wt).

All measured as phenothiazone after oxidation.
Drawn from recalculated data (Refs. 76,79).

a small proportion of the administered dose; at peak values the amount of drug in the total blood volume, assuming equal distribution, only accounts for about 1 to 2% of the total body burden.

A detailed investigation into radioactive blood levels has been carried out in the guinea pig following oral ingestion of (^{14}C)-labelled phenothiazine⁶³. A semilogarithmic plot of the data gives a curve (Fig. 4) which is indicative of a three-compartment model (absorption, distribution, elimination). Only a few observations fell into the delayed portion of the curve where absorption became negligible; nevertheless, the biological half-life can be estimated as about 24 hours. Identical treatment of the scanty data available for the sheep⁷⁹ gives a similar curve and an approximate half-life of 15 to 18 hours.

Quantitative studies of secretion by way of the less important routes of elimination are unfortunately even more limited. Biliary secretion levels in the cow and guinea pig of total phenothiazine and phenothiazone and of radioactivity respectively show similar patterns (Fig. 5). An initial peak occurs around 10 to 12 hours after dosing in the cow and at 13 to 14 hours in the guinea pig to be followed by a second larger peak 2 to 3 hours later in both species. No drug was detected in samples of bile collected from cows at 120 hours after dosing⁸¹.

Several studies have shown that coloured phenothiazine derivatives are secreted in the milk from cows, goats and sheep, but only one study with sheep provides sufficient serial collection data to construct a cumulative secretion curve (Fig. 6). This curve has a similar shape to that for urine with a maximum secretion rate occurring in the 12 to 24 hour period. However, although the amount of drug secreted is sufficient to colour the entire milk yield it can be shown here to account for only 0.2% of the total dose (ie. 76 mg in 38 g) in 3 days⁷⁹.

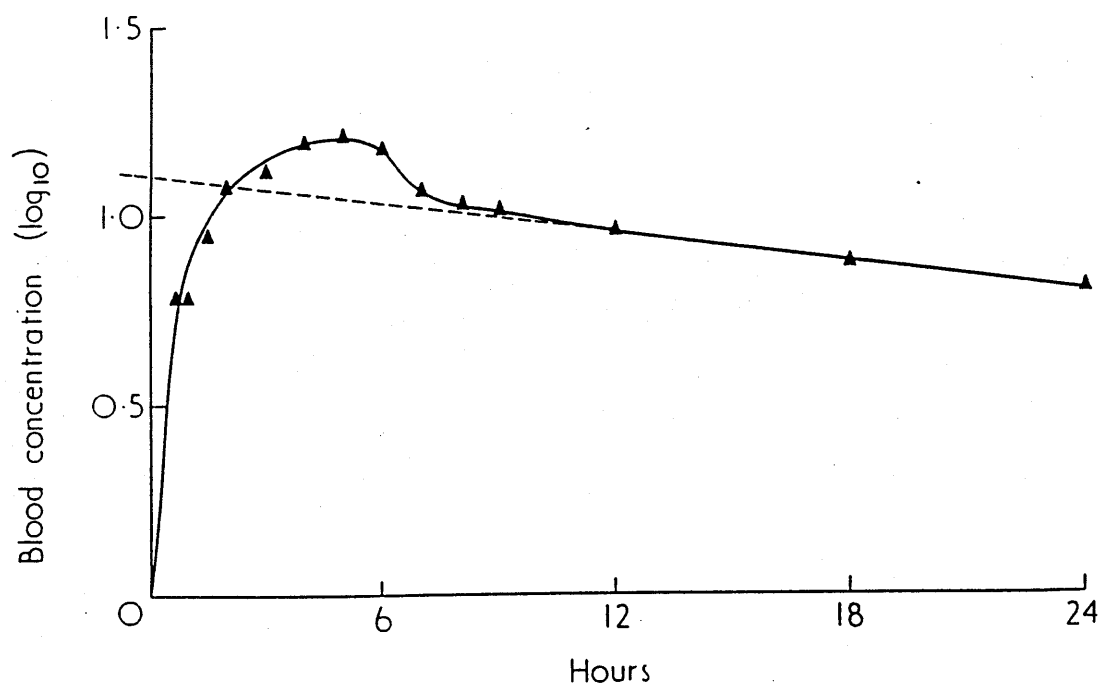


Fig. 4. Semilogarithmic plot of blood radioactivity levels in the guinea pig after oral administration.

Each point represents the mean of four animals (dose 150mg/kg body wt).

Drawn from previously unpublished data (Ref. 63).

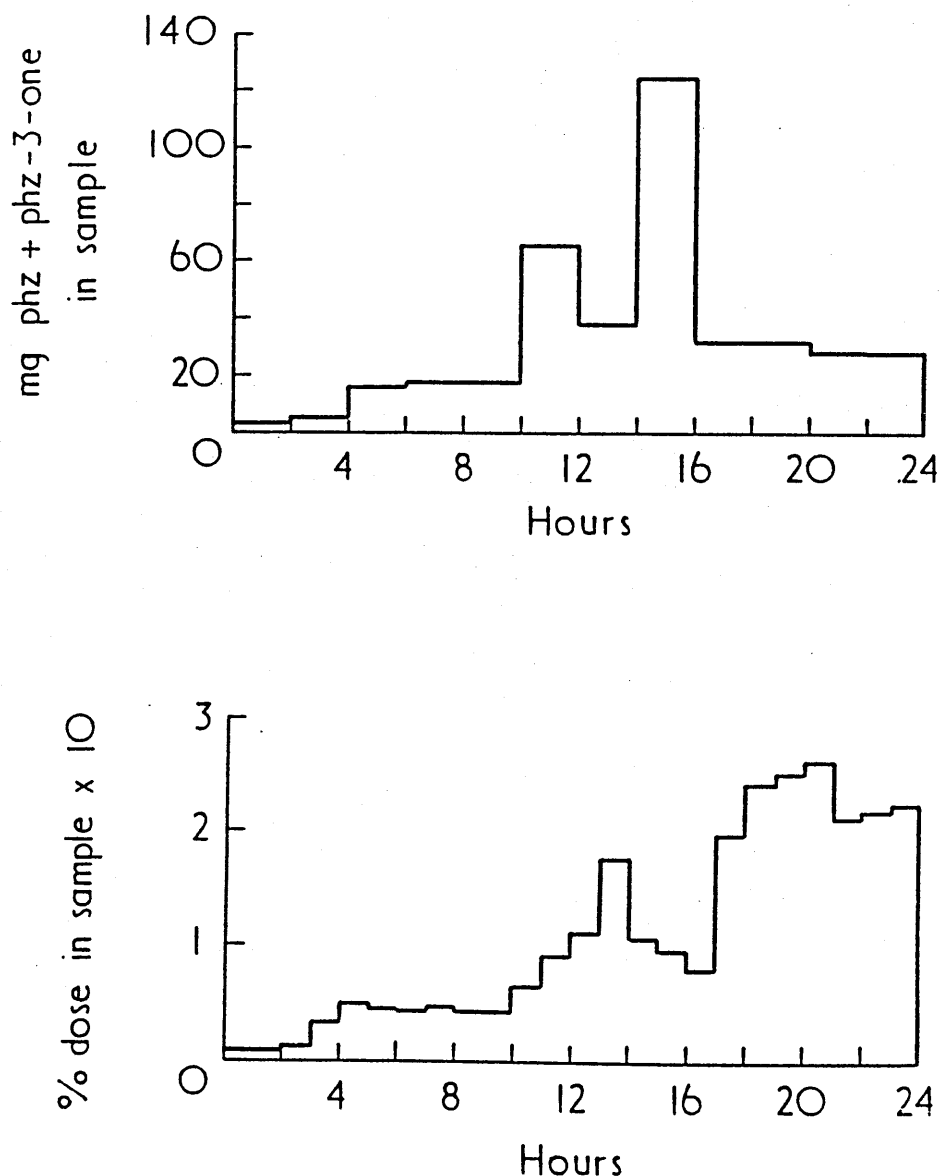


Fig. 5. Biliary secretion of orally administered phenothiazine.

TOP. Cow - bile samples collected during post mortem of 18 dairy cows at varying times after dosing. Specific doses not given but range from 420mg to 1.47g/kg body wt. Drug was measured spectrophotometrically after bromination and after acid hydrolysis. Drawn from recalculated data (Ref. 81).

BOTTOM. Guinea pig - bile samples collected continuously via an indwelling cannula. Dose 150mg/kg body wt, ^{3}H drug. Drug was measured radioactively. Each value represents the mean of four animals. Drawn from previously unpublished data (Ref. 63).

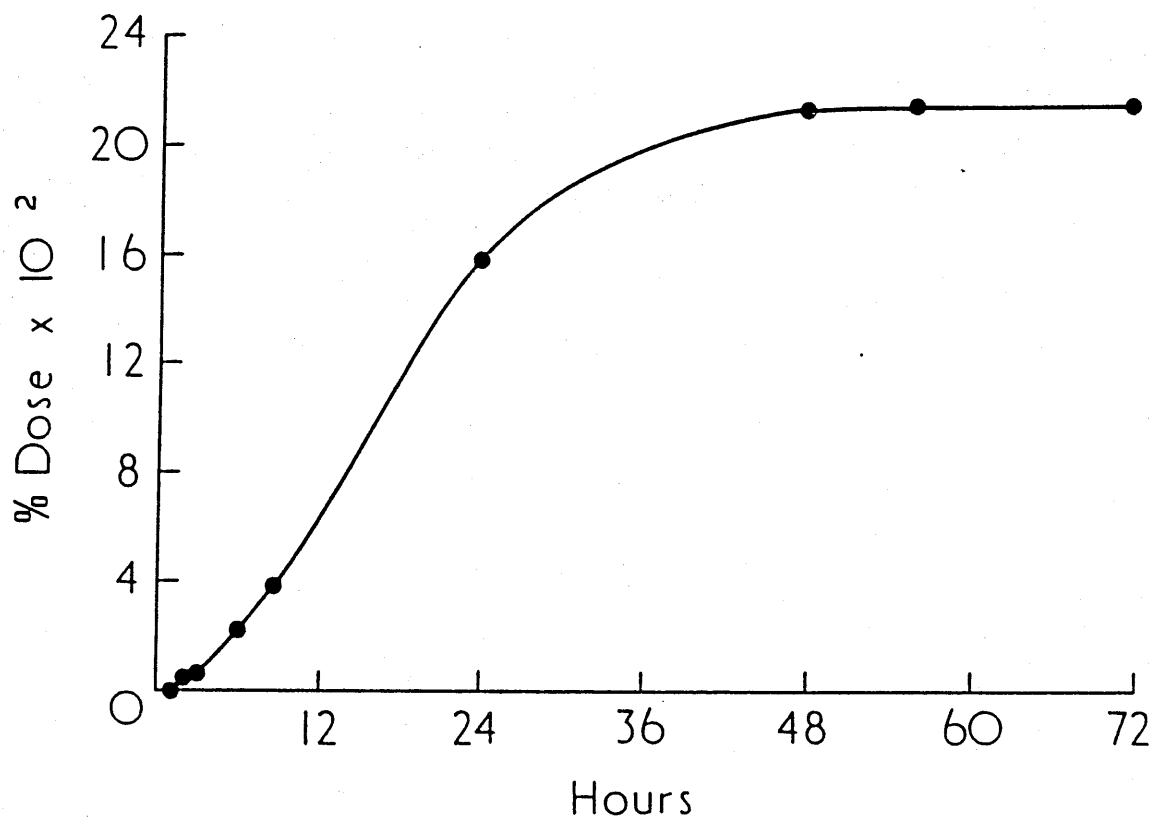


Fig. 6. Cumulative secretion of orally administered phenothiazine in sheep milk.

Graph represents values from one animal (dose 38.0g; body wt not specified).

Measured as phenothiazone after oxidation.

Drawn from recalculated data (Ref. 79).

3. ADVERSE REACTIONS

A. INTRODUCTION

An insight into the mechanism of interaction of a drug with the biochemical systems within a living organism may be gained by a detailed examination of its effects, both beneficial and deleterious, for any perceptible effect which occurs must have an underlying physiological dysfunction resulting from the disturbance or modulation of a biological event. The work undertaken into the mode of action of phenothiazine on intestinal parasites is discussed in detail later. The second avenue of approach, dealt with below, is through the examination of the various side effects, the unwanted reactions, which the drug produces in various animals. However, before this avenue can be fruitfully explored it is necessary to establish what specific adverse reactions take place after phenothiazine administration.

Unfortunately, the effects of the drug are quite variable; not only between the several species treated but sometimes between individuals belonging to the same species. Animals have died after receiving the customary therapeutic dose, whereas others have survived many times this amount. The various species have been placed in order of increasing sensitivity to phenothiazine toxicity (dogs, birds, rabbits, goats, sheep, cattle, pigs, horse, man) and it has been recommended that small gradual doses of the drug be given rather than larger doses ¹²⁶, although continuous treatment may be more insidious in its harmful effects ⁴⁹ and it is almost certain that phenothiazine would be toxic to all animals if the acute or chronic dosage is too high ^{127,128}.

Undoubtably a variety of factors play a role in determining the susceptibility of any animal although idiosyncrasy alone does not appear to be a satisfactory explanation for all cases of poisoning. Toxic manifestations often tend to occur in groups, suggesting some localised environmental or genetic influence. One such example uncovered whilst investigating the anthelmintic efficiency of phenothiazine found that just under one quarter of the 89 patients treated showed some form of adverse reaction and that three who developed anaemia were sisters ¹²⁹. Another report states that the sister of a young girl admitted to hospital with acute haemolytic anaemia after phenothiazine treatment had also suffered a very similar but milder attack ¹³⁰. One thing that is apparent is that the young of a species are generally more prone to adverse reactions than adults.

B. EFFECTS ON THE BLOOD ELEMENTS

The most widely reported side effect of phenothiazine therapy is acute haemolytic anaemia and its sequelae. The severity of this condition can vary between being slight and transient to fatal in its outcome and has been reported as occurring in many species including the mouse ¹³¹, rat ^{132,133}, rabbit ¹³², dog ^{125,134,135}, pig ^{136,137}, cattle ¹³⁸, horse ^{76,135,139-147}, and man ^{42,110,111,130,148-166}. Anaemia has also been demonstrated in chickens after phenothiazine administration but this may have been due to the catching and confining of the birds ^{167,168}.

It is difficult to obtain a reliable measure of the incidence of haemolytic anaemia within a species or population as very many articles only cite those individuals experiencing side effects and do not contain controlled studies. Nevertheless, the literature contains several instances where horses have been efficiently dosed without

the appearance of any clear signs of intoxication ^{49,169-173} and it is certain that many thousands of horses throughout the world have been successfully treated without adverse reaction. A few reports exist where the efficiency of phenothiazine therapy in humans has been studied and the number of subjects developing any changes within their blood profile have been cited (Table 9). The observed overall incidence of 18% is similar to the 13% quoted by Johnstone and Grant ^{130,153} for their review of 58 patients, although the later included nine patients treated by one investigator who reported no side effects ⁴⁷. However, a wide variation is seen to exist between the various studies (Table 9) and the quoted mean value may be an underestimate. Miller and Allen have stated that a slight but definite anaemia occurred in about half of a group of 73 children, aged between 4 and 12 years, treated with phenothiazine ¹⁵⁰ and other workers have also mentioned a transient but measurable anaemia in about half of their subjects ^{152,155}. Nevertheless, severe haemolytic anaemia has been cited ^{149,153} and this, coupled with the report of a death due to phenothiazine poisoning ^{174,175} resulted in the general feeling that its use in man was unjustified.

In addition to the general picture of a decreased haemoglobin content, decreased red blood cell count and an increase in the percentage of circulating reticulocytes (rat ¹³², dog ¹³⁴, man ⁴²), anisocytosis (considerable variation in size)(dog ¹⁷⁶, horse ¹⁴⁷) and poikilocytosis (irregular shape)(horse ¹⁴⁷) have been observed as well as a polycythaemia (increase in number of red blood cells above normal), the later occurring in lambs given frequent doses of phenothiazine ¹⁷⁷. Effects on white blood elements include neutrophilia (dog ^{134,176}), low polymorphonuclear cell counts (man ¹⁴⁹) and a general predisposition to agranulocytosis (sheep ¹⁷⁷, man ¹⁶⁴). Other signs reported include haemoglobinuria (horse ^{140,141}), albuminuria (horse ^{140,141,147}, goat ¹⁷⁸),

Table 9. Incidence of haemolytic anaemia in subjects undergoing phenothiazine treatment.

Number of subjects examined	Subjects developing haemolytic anaemia		Reference
	number	percent incidence	
23	9	39.1	152 Bercovitz <u>et al.</u> , 1943
8	3	37.5	149 Graham, 1942
19	3	15.8	42 DeEdds <u>et al.</u> , 1939
28	3	10.7	148 Hubble, 1941
34	2	5.9	155 Kuitunen-Ekbaum, 1946
<hr/> 112	<hr/> 20	<hr/> 17.9	

an increase in lactic acid and bilirubin in the blood stream (horse ¹⁷⁹), jaundice (dog ¹³⁴, man ¹⁸⁰), nephritis and hepatitis (man ^{149,166}).

Post mortem examination has shown severe injury to the urinary system (horse ⁷⁶) together with pathological lesions in the kidney (sheep ¹⁷⁷) including changes in the tubules and Malphigian corpuscles (horse ¹⁸¹) and coagulated masses in the bladder, kidney hilus and ureters (horse ¹⁸²). A mention of organ congestion, fatty degeneration and advance cloudy swellings of the liver, interstitial lesions of the heart (horse ^{181,183}) and enlargement of the spleen (horse ¹⁸², weasel ¹⁰⁰) heart, liver and kidneys (horse ¹⁴⁴) has also been made. Most, if not all, of these changes may well be secondary to the haemolytic anaemia present.

C. NEUROMUSCULAR PROBLEMS

Loss of equipoise and power of coordination in movement with difficulty in walking, staggering gait, muscle weakness and general paralysis of the hind quarters have been observed with pigs ^{136,184-187}, horses ^{144,188,189} and cattle ¹⁹⁰ undergoing phenothiazine treatment. The phenomenon is the most dramatic in pigs where some animals are so badly affected that they are unable to move, lying prostrate with opisthotonus (a tetanic spasm in which the spine and extremities are bent with convexity forwards) being observed in a few animals ¹⁸⁵. The condition can be fatal ¹⁸⁵.

D. PHOTOSENSITIZATION

The phenomenon of photosensitization has been frequently described in man. The reaction consists mainly of an itchy rash and inflammatory dermatitis resembling sunburn, developing after several hours direct exposure of the skin to sunlight. The reported cases have either followed the direct contact of the skin with a spray containing phenothiazine or a dust of the chemical ¹⁹¹⁻¹⁹⁴ or after

the ingestion of phenothiazine (or in one case thionol) ^{155,195}, suggesting that both a topical irritation and a sytemic mechanism may be in operation. Nevertheless, it is generally appreciated that both the drug and direct sunlight must be present for the reaction to occur; ranchers in Texas avoid this irritation by dosing their herds at night ¹⁹⁶.

Other animal species also show photosensitization but do not react with the same frequency. White animals, or animals with white spots, may react whenever unpigmented parts are exposed to the sun. Animals having a general pigmentation may only show an inflammation of the cornea and associated signs such as lacrimation and oedema of the eyelids. The keratitis may develop into opacity of the cornea and eventual ulceration leading to temporary or in some cases permanent blindness. Such reactions, in varying degrees, have been reported as occurring in pigs ¹⁹⁷, sheep ¹⁹⁸⁻²⁰¹, cattle ^{59,202-205}, goats ²⁰⁶ and pheasants ²⁰⁷.

E. OTHER REACTIONS

A variety of other adverse reactions have been cited in the literature and have been linked with phenothiazine therapy. These include shock resembling anaphylaxis after the oral administration to a horse ²⁰⁸, increased thirst in pigs ¹⁸⁴ and horses ⁷⁶, enteritis diagnosed at post mortem in a horse ²⁰⁹ and gastrointestinal irritation in man ¹⁵⁶. Colicky pains, diarrhoea and constipation have also been reported together with impaction of the colon in the horse ¹⁸². Subnormal temperatures have been recorded in cattle ¹⁹⁰ and pigs ²¹⁰ and fever in man ¹⁵⁵ together with a scarlet rash and slight oedema about the eyes and hands ^{155,156}. Sores and severe dermatitis resembling scabies have been described in pigs ^{185,210} and acute dermatitis with exfoliation has been reported in man ²¹¹. Abortion in sheep ²¹² and an

increased incidence of stillborn lambs has also been cited ^{213,214}.

4. MODE OF ACTION

Before the specific aspects of the mode of action of phenothiazine and the possible mechanisms by which the major adverse reactions, now established as haemolytic anaemia, neuromuscular incoordination and photosensitization can be examined in detail it is important to realise that several factors other than the direct biochemical effects of phenothiazine or its metabolites may influence the overall picture of therapy and toxicity in a given animal. These will be considered first before pursuing explanations which evoke biochemical interactions.

A. CONTRIBUTING FACTORS

1). Impurities.

It is possible that poisoning resulting from the administration of phenothiazine is due to impurities within the preparations. In eastern Siberia during March 1955, 1350 foals were each given 40 to 60 grams of phenothiazine as a prophylactic measure. Thirty of the animals became ill within two or three days after dosing and 22 (1.6%) subsequently died. On post mortem examination an alkaloid was detected in the organs and a compound, when recovered from the stomach contents of the foals, was shown to be poisonous to rabbits. It was proposed that this was derived from the phenothiazine and was responsible for the poisoning but it was not stated whether or not the alkaloid(s) was detectable in the phenothiazine before administration ^{215,216}. The possibility that the fatal compound was a plant alkaloid taken in whilst feeding must also be considered. In another study involving

horses undergoing phenothiazine treatment it was concluded that the upset in the blood picture was caused to a large extent by impurities, mainly diphenylamine ²¹⁷. Diphenylamine is employed in the synthesis of phenothiazine and is itself used topically in the treatment of screwworm infestations. Large oral doses of diphenylamine (up to 30 g) given to sheep have been shown to produce similar but more severe lesions than those occasionally found with phenothiazine ²¹⁸.

Several workers have shown that commercial phenothiazine reduces the uptake of radioactive iodine by the thyroid gland ²¹⁹⁻²²³ but did not induce hypothyroidism ²²⁴. Blood levels of iodine increased in sheep given phenothiazine ²²⁵ and the thyroid glands of treated animals were shown to contain nearly twice as much iodine as the glands from control animals, although there was no significant increase in the mean wet weight ²²⁶. Such effects were not seen when purified phenothiazine was administered and it is assumed that they were due to iodine or iodide present as an impurity in commercial phenothiazine ^{221,223,227}; iodine being used as a catalyst in its synthesis. Other workers have suggested that another factor, much less potent than iodine, was also responsible for a slight depression of thyroid uptake of radioactive iodine, but its identity has not been stated ²²⁸.

Other compounds, such as methylamine, can be added in small amounts (0.3 to 1.0% w/w) to phenothiazine to prevent its oxidation in bright sunlight, especially when in the presence of a finely divided inert carrier, which leads to the compound acquiring a greenish-brown tint. It is possible that these trace contaminants, either remaining from the manufacturing process or being deliberately added afterwards, may add to the toxic reactions observed, especially as such large doses of phenothiazine are routinely given. However, studies employing purified phenothiazine have shown that adverse reactions still occur.

2). Physical effects.

The post mortem finding of an impacted colon in the horse together with many reports of constipation after phenothiazine treatment suggest that a large proportion of the dose, which may be up to 1 kg in some horses ²²⁹, is remaining within the lower gut lumen bringing about bowel dysfunction and leading to colicky pains and other diffuse symptoms of general ill health reported in the literature ^{144,147,182,190}. It is also possible that a large amount of virtually insoluble phenothiazine within the colon will interfere with water reabsorption (diarrhoea has also been reported) leading to an increased thirst as observed in pigs and horses ^{76,184}, dehydration reported in ponies ²³⁰ and impairing the urinary excretion of the drug and its metabolites, perhaps encouraging the formation of insoluble precipitates within the kidney. When lambs were subjected to a process of slow dehydration they were far more susceptible to phenothiazine toxicity (kidney lesions) than those not dehydrated and this is strongly suspected as being a factor precipitating mortality in New Zealand sheep ^{218,231}. Phenothiazine itself has been shown to possess a mild diuretic action and would therefore accentuate the state of dehydration ²¹⁸.

3). Nutritional factors.

Animals suffering from infection will be in a state of general ill health before phenothiazine administration and dietary deficiencies, whether owing to inadequate feeding in ill health or to insufficient nutriment value of fodder, especially over the winter months, may give rise to a general predilection to toxicity ^{144,232}. Experience with the mass treatment of horses in the USSR indicated that toxic reactions only occurred in stabled horses during the winter months and were thus attributed to nutritional deficiencies ²³³. However, this point is controversial with some workers reporting that horses in good or fair

condition are more likely to show severe anaemic reactions than horses in thin condition ²³⁴, whereas others suggest that emaciated or aged subjects are more at risk and that a high protein diet can protect, at least in part, against the haemolytic effects of phenothiazine ¹⁴⁵. Indeed, one dog on a diet deficient in vitamin B experienced a greatly intensified anaemia and jaundice than that normally induced by phenothiazine treatment although the addition of vitamin B complex to normal diets did not prevent the anaemia ¹³⁴. In chickens phenothiazine has been shown to increase the deposition of vitamin A in the livers as well as increase the growth rate when added to vitamin E deficient diets containing 10% cod liver oil ²³⁵. The significance of these later observations and complex interactions are not known.

B. MECHANISM OF ANTHELMINTIC ACTION

'A completely satisfactory explanation of the mechanism by which phenothiazine eliminates nematodes has defied research workers for over twenty years' ²³⁶.

That statement was delivered in 1962 after phenothiazine had enjoyed two decades as the most efficient and widely used anthelmintic on earth. With the decline of its popularity shortly afterwards little additional information has been gleaned and the position of understanding today shows little advance over that achieved in the '60's.

Three features of the action of phenothiazine which are difficult to explain are the requirement of a relatively large dose for full activity, the greater efficiency against parasites of the abomasum (fourth division of the ruminant stomach) caecum and colon compared with those of the small intestine and the mechanism by which the parasites are removed.

Investigations with phenothiazine and its derivatives suggest that the compound is not converted into an active anthelmintic by metabolism¹⁰² and it is therefore assumed that the large doses are required to ensure that the parasite absorbs toxic amounts of the unchanged drug. Although the compound has a very low aqueous solubility (1.25 p.p.m.) it is generally accepted that it enters the parasitic nematode through the cuticle¹⁰⁴ and thus must be in solution before absorption can occur. The rate of solution will depend upon the total available surface area of the drug and if this falls below a critical level the concentration of phenothiazine in solution, which is constantly being removed by absorption into the host, will be too low to remove the parasitic nematodes even though solid phenothiazine may still be within the gut lumen. This hypothesis is supported by observations showing that the anthelmintic efficiency increases as the particle size of the administered phenothiazine decreases²³⁷⁻²³⁹.

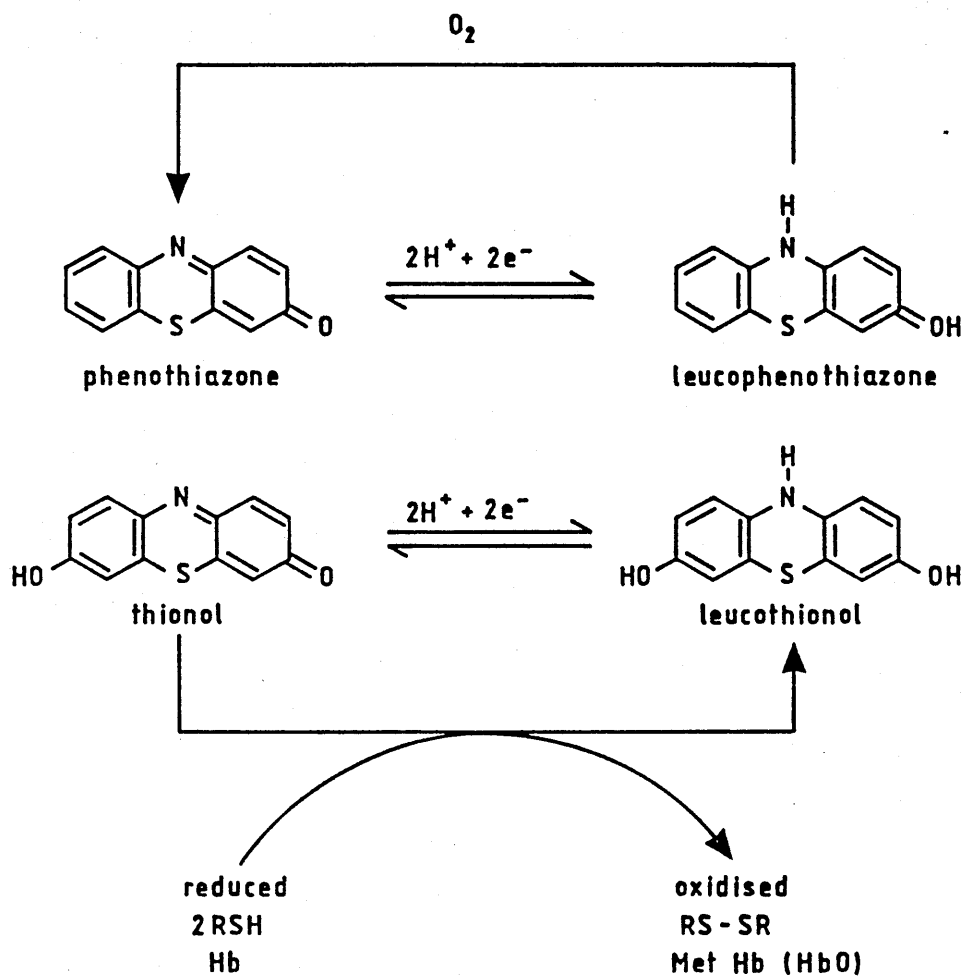
As a corollary it seems reasonable that the anthelmintic efficiency of phenothiazine in the various parts of the gastrointestinal tract is related to the relative differences in the drug concentration and the time for which they persist. Since the small intestine is the site most highly specialised for absorption it is to be expected that in this region the balance between absorption by the host and solution from the solid state will be the most unfavourable for anthelmintic action²³⁶.

The third point of interest is the way in which phenothiazine eliminates the parasitic nematodes. As previously mentioned, this appears to be a property of phenothiazine itself and not a metabolite¹⁰². No anthelmintic activity was noticed when phenothiazone and thionol were administered to goats heavily infected with parasitic worms⁴⁹ and the dosing of phenothiazone to sheep had no effect on nematodes previously shown to be removable by phenothiazine⁶⁴. Also, metabolism

of phenothiazine within the nematode does not seem to occur; the phenothiazine content of nematodes taken from treated animals did not decrease when the worms were kept in a drug-free medium for up to 24 hours, suggesting that once phenothiazine has entered the parasite it has great difficulty in leaving¹⁰²⁻¹⁰⁵.

Phenothiazine does not exert a lethal action on the parasite as worms eliminated from phenothiazine treated animals are alive and can be maintained in vitro for just as long as parasites from untreated animals²³⁶. The only definite effect so far demonstrated both in vivo and in vitro is the inhibition of egg laying which is thought not to be related to any specific drug action on the nematode reproductive system but an overall change in the well being of the adult²³⁶.

Metabolic studies have shown that phenothiazine has the ability to form two redox systems (Scheme 3) and it has been postulated that such systems may prevent the operation of an oxygen transfer mechanism essential to helminth metabolism and thus be related to its anthelmintic activity. Circumstantial evidence for this view comes from structure / activity studies which showed that phenothiazine derivatives with substituents in both the 3- and 7-positions, which precludes the formation of such redox systems, are devoid of anthelmintic activity. Only a few compounds with substituents in the 3-position displayed any activity and an apparent association was found between the anthelmintic activity of a compound and the ability to form semiquinones with oxidation potentials in the region of 550-850 mV²⁴⁰. However, despite much theoretical reasoning that such systems may play a role in the action of phenothiazine, the action of the drug or of a metabolite on any nematode redox system which could account for the anthelmintic action remains to be demonstrated.



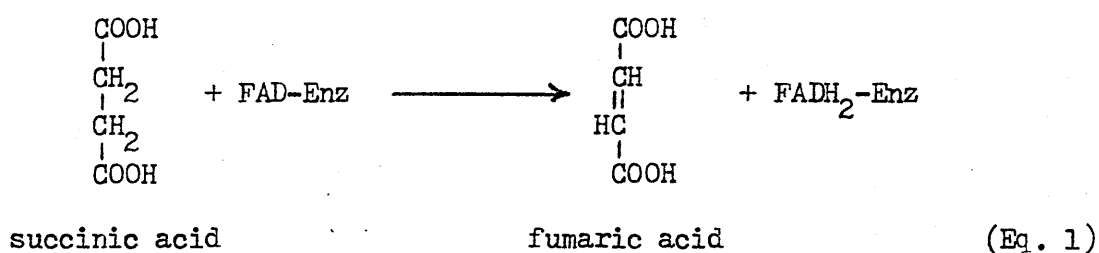
Scheme 3. Redox systems present in phenothiazine metabolites and their possible oxidative interaction with tissue components.

C. INHIBITION OF ENZYME ACTIVITY

Over the years from 1940 to 1955, the time when phenothiazine enjoyed immense popularity, several workers reported that the compound and its metabolites displayed inhibitory effects towards the in vitro activity of a wide variety of enzyme systems. This data has been collated and contains enzyme systems belonging to the functional categories of oxidoreductases, hydrolases and transferases (Table 10). Speculation as to the means of inhibition has been made but there appears to be no simple unifying characteristic or property possessed by these diverse enzyme systems to make a universal inhibitory mechanism possible and it is probable that several modes of action are involved.

1). Sulphydryl interaction.

Investigations have revealed that the activity of rat liver succinic dehydrogenase, an enzyme of the tricarboxylic acid cycle which catalyses the oxidation of succinic acid to fumaric acid (Eq. 1), is inhibited by quinones and related compounds and it has been suggested that the sulphydryl groups of the enzyme may be involved ²⁴⁹.



A similar type of mechanism was advocated for the inhibitory effects of phenothiazine derivatives that have quinone-like structures, eg. phenothiazone. It was further assumed that these derivatives would act on the sulphydryl groups of glyoxylase (aldoketomutase), an enzyme system which catalyses the intramolecular oxidation-reduction of methylglyoxal (pyruvic aldehyde) to lactate and employs glutathione as a specific coenzyme in this dismutation (Eq. 2), and thus bring

Table 10. Inhibitory effect of phenothiazine and its metabolites on enzyme systems.

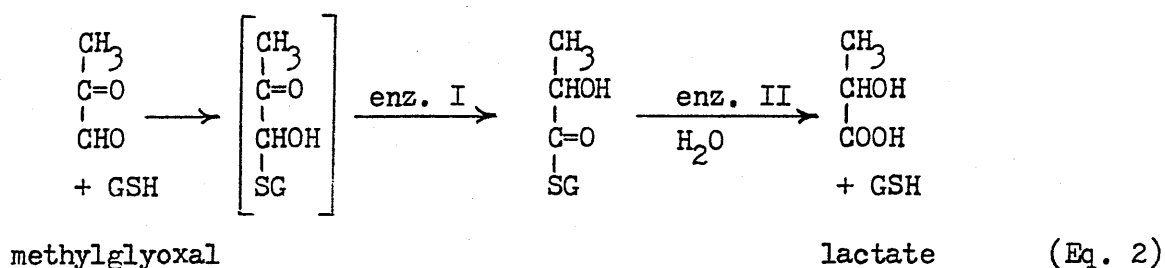
Enzyme activity	Source of enzyme activity	Inhibitor ^a						Literature reference
		A	B	C	D	E	F	
<u>1). Oxidoreductases</u>								
<u>oxidase</u>								
cytochrome oxidase	beef heart		-	+	+			241, 242
	cockroach coxal muscle ^b	+						243
	rat liver mitochondria	-	-	-	-	-		244
<u>aerobic dehydrogenase</u>								
D-amino acid oxidase	pig kidney cortex		-					242
<u>anaerobic dehydrogenase</u>								
lactic dehydrogenase	yeast		+	^c				242
succinic dehydrogenase	beef heart		+	-	+			242, 245, 246
	rat liver mitochondria	+	+	+	+			244
<u>hydroperoxidase</u>								
catalase	guinea pig liver	-	+	-	+	+	+	241, 242, 245
<u>2). Hydrolases</u>								
<u>esterases</u>								
cholinesterase	horse serum		+			-		245, 247
lipase	horse serum		-					247
phosphatase (alkaline)	kidney		-					247

Table 10 cont.

Enzyme activity	Source of enzyme activity	Inhibitor ^a						Literature reference
		A	B	C	D	E	F	
<u>amidases</u>								
urease	jack beans (?)			+				242
<u>lyase</u>								
glyoxalase	human erythrocytes	+		+				120
	rabbit erythrocytes	+	+	+				120
3). <u>Transferase</u>								
<u>transphosphorylase</u>								
hexokinase	rat brain ^d			+				248

- ^a inhibitor substance; A phenothiazine sulphoxide, B phenothiazine, C phenothiazone, D leucophenothiazone, E thionol F leucothionol. - no inhibition, + inhibition. Absence of a sign : not tested.
- ^b ascorbate added as substrate completely reduced the added phenothiazone to leucophenothiazone and partially reduced the thionol.
- ^c phenothiazone added was completely reduced to leucophenothiazone.
- ^d this tissue was homogenised in the presence of fluoride to depress apyrase activity.

about inhibition of its activity. This was found to be the case but inhibition still occurred even in the presence of a large excess of glutathione which is known to act as a protective agent for these sulphydryl groups. This was in direct contrast to the observation made with p-chloromercuribenzoate, which is known to inhibit enzymic activity by reacting with sulphydryl groups, as this latter compound lost its inhibitory properties in the presence of excess glutathione¹²⁰. Undoubtably some additional explanation is required.



2). Redox systems.

The existence of two oxidation-reduction systems amongst the metabolites of phenothiazine (phenothiazone / leucophenothiazone ; thionol / leucothionol ; see Scheme 3) has prompted the suggestion that the inhibition of succinic dehydrogenase may be related to the high redox potentials possessed by these systems. Phenothiazone has an E'_0 value of +0.13 volts at pH 7.3 and the thionol system an E'_0 of 0.16 volts at pH 7.0, both of which are higher than that of the fumarate-succinate couple ($E'_0 = 0.03$ v., pH 7.0)^{28,73,75,242}. It is possible that in general such redox reactions may be related to the observed enzyme inhibitions. However, the reduction potential values were determined in vitro and apply only to 50% reduced systems at the given pH values and it is far from certain that these represent the systems as they actually occur in living cells. The variation of pH in vitro is known to markedly affect the reduction potential of the thionol system (Table 11)^{28,73}.

Table 11. Reduction potentials of thionol at various pH values.

pH	E'_O	pH	E'_O
1.42	+0.47	6.57	0.19
1.85	0.46	6.84	0.17
2.65	0.41	7.00	0.16
3.47	0.39	7.14	0.15
4.03	0.35	7.44	0.13
4.47	0.30	7.78	0.11
4.99	0.27	8.70	0.07
5.53	0.25	9.90	-0.004

Values taken from references 28,73.

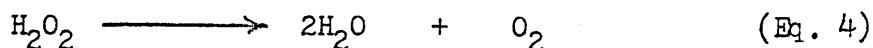
The inhibitory effects seen with phenothiazine may well be due to oxidation within the incubation medium to form phenothiazone / leucophenothiazone. It has been shown that phenothiazine completely inhibited the activity of cytochrome oxidase, the terminal member of the cytochrome chain capable of reducing oxygen (Eq. 3), in cockroach muscle but only after one hour of pre-incubation during which time only partial inhibition occurred ²⁴³. The death of cockroaches owing to the inhibition of cytochrome oxidase has also been speculated but no experimental details were given ³⁸.



It is known that cytochrome oxidase is readily auto-oxidizable only in the presence of cytochrome c, probably forming a 'cytochrome a (oxidase) - cytochrome c complex' before activity can occur. Spectrophotometric studies have shown that leucophenothiazone can reduce cytochrome c but it was not certain whether or not this reaction was reversible. It is possible that the inhibition of cytochrome oxidase activity occurs by the maintenance of cytochrome c in its reduced state ²⁴¹.

3). Other mechanisms.

The inhibition of catalase activity, a hydroperoxidase enzyme which breaks down hydrogen peroxide with the evolution of molecular oxygen (Eq. 4), by the hydroxy-phenothiazines has been attributed to the phenolic -OH group which possibly forms covalent complexes with the active iron atoms of these haemoprotein catalysts ²⁴².



It might be expected that a molecule of phenothiazine's structure would intercalate by van der Waals bonding to other aromatic ring systems thereby binding to the porphyrin / corrin ('tetrapyrrole') ring structure of haemoproteins or binding to protein residues and bringing about disruption of their tertiary structure. This could be a common mechanism by which phenothiazine and its derivatives are able to inhibit the activity of such a functionally diverse range of enzyme systems. Indeed, phenothiazine and thionol have been shown to bind to serum albumin and nucleic acids¹²¹ and recent n.m.r. studies have suggested that substituted phenothiazines can bring about changes in the protein configurations in red blood cell membranes (haemolytic anaemia ?) and so this remains a real possibility²⁵⁰.

Since the mid 1950's no further work has been undertaken to elucidate the mechanism(s) of phenothiazine-induced enzyme inhibition and the suggestions cited above are based on very little if any evidence other than the observation of inhibition with a given compound.

Some of these effects (Table 10) may be related to the observed side-effects of the drug but extreme caution must be applied when attempting to extrapolate data obtained from in vitro experiments to the in vivo situation.

D. PHENOTHIAZINE-INDUCED ADVERSE REACTIONS

1). Haemolytic anaemia.

As with all the toxic effects ascribed either directly or indirectly to phenothiazine therapy there is a marked variation in the susceptibility of different species to its haemolytic manifestations. Whilst man, horse and dog are most prone to this adverse reaction, no

significant blood changes have been reported in the guinea pig or golden hamster ¹³⁴.

(i). Direct action.

The observation of an increase in erythrocyte fragility preceeding phenothiazine-induced anaemia in dogs was interpreted as being due to the direct action of the compound and its derivatives on erythrocytes, subsequently producing haemolysis ¹³⁴. Experiments with the phenothiazine derivatives promazine and chlorpromazine have demonstrated that in vitro incubation with human and dog erythrocytes resulted in a rapid haemolysis and that the intravenous administration of these substances in the dog produced similar results. This effect was thought to be due to an increase in the permeability of the red cell membranes to water or macromolecules ²⁵¹. It has also been shown that substituted phenothiazines induce apparent conformational changes in the protein phase of erythrocyte ghosts ²⁵⁰ and that chlorpromazine and its analogues antagonise calmodulin-stimulated ($Mg^{2+} + Ca^{2+}$)-ATPase activity (possibly necessary for microtubule integrity) in similar preparations ²⁵². Although no such detailed work has been undertaken with phenothiazine itself and it must be remembered that these N-substituted phenothiazines possess completely different physical, chemical and physiological properties from the parent compound, it is quite possible that phenothiazine may interact with the lipid phase of the membrane owing to its high lipid-solubility or bind to the protein phase by van der Waals interaction, as has been shown for the oxidation products phenothiazone and thionol ¹²¹, thereby bringing about changes in molecular orientation and membrane permeability. Indeed, experiments previously reported in detail have shown that phenothiazine and its derivatives become bound to the membranes of erythrocytes but do not enter into the cells ^{55,63,80,119-121}.

However, studies involving in vitro incubations of human and horse erythrocytes (two susceptible species) with phenothiazine metabolites failed to reveal any haemolytic action ^{120,253}. It is possible that these compounds may damage the erythrocyte in such a way that they are then selected for removal by the reticuloendothelial system. If so, then subtle changes in the surface characteristics or alterations in shape or plastic properties of the cell may be sufficient to initiate this process. Overstimulation of the spleen and destruction of erythrocytes by hyperactivity has also been proposed ⁷⁶ and descriptions of splenomegaly lend some support to such a hypothesis ^{140,254}.

(ii). Indirect action.

In 1942 it was reported that the in vitro haemolysis of horse erythrocytes by saponin and lysolecithin was rapidly accelerated in the presence of phenothiazone or the potassium leucophenothiazone sulphate conjugate; erythrocytes from sheep (a more resistant species) required three times the concentration of these chemicals to produce the same rate of haemolysis as with horse cells. A concentration of 3×10^{-4} M of the conjugate has been detected in horse plasma after phenothiazine treatment which, in vitro, was sufficient to cause a very marked acceleration of lysolecithin induced haemolysis ²⁵³. Lysolecithin, which is a normal component in mammalian circulation particularly the horse, and saponin are surface active agents ('detergents') and powerful lytic agents. The mechanism by which the phenothiazine derivatives accelerate this haemolytic process is not known but it is believed that the red blood cells are being constantly subjected to lytic tendencies and the presence of such a drug hastens this process of lysis, perhaps by an independent but additive effect on the cell membrane (see above).

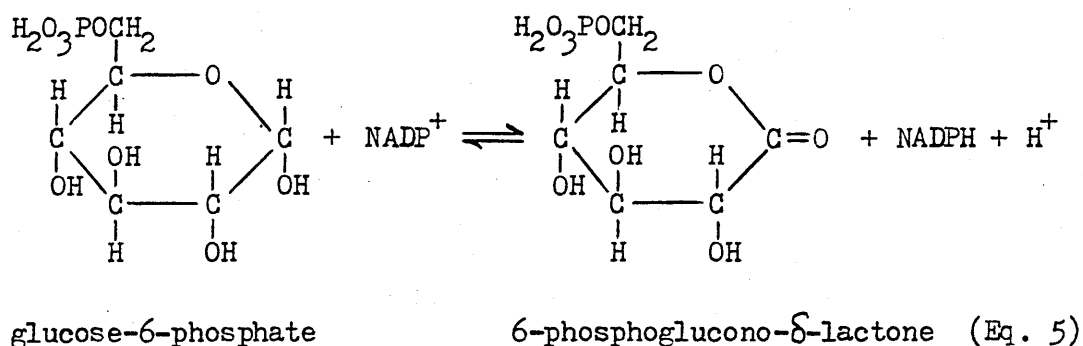
It is known that most haemolytic drugs are actually or potentially aromatic compounds capable of forming reversible oxidation-reduction systems ²⁵⁵. Phenothiazine is known to be metabolised in vivo to form two such redox systems (see Scheme 3). Such compounds have the property of reacting with oxygen probably to form oxidant intermediates or free radicals which are capable of oxidising haemoglobin and other intracellular components. Small amounts of methaemoglobin have been detected when erythrocytes were incubated with phenothiazone ²⁵³. The possibility of photodynamic haemolysis, such as seen with rose bengal or eosin where damage to the cell membrane is caused by energy transfer from a radiation induced excited state of the absorbed drug molecule to the membrane ²⁵⁶, has been discounted since in vitro incubations of horse erythrocytes with phenothiazone under irradiation produced no detectable haemolysis ²⁵³.

Free intracellular thiols, of which glutathione is the major source, are also oxidised or destroyed and with the depletion of the cell's reducing capacity the continued integrity of the erythrocyte is severely threatened ²⁵⁷⁻⁹. The oxidation or blocking of red cell thiols is known to be associated with increased membrane permeability and haemolysis in vitro ^{260,261}. This increased susceptibility of the cell to oxidative stress leads to the spontaneous or accelerated oxidation of haemoglobin and its subsequent loss of normal configuration. The denatured haemoglobin then undergoes polymerisation and is deposited as small dense bodies beneath the red cell membrane. These inclusion bodies (Heinz bodies) are present in small numbers in some normal erythrocytes as they approach the end of their useful life span (about 120 days) and consequently this 'premature ageing' induced in the cell may be a signal for their selective removal by the reticuloendothelial system.

(iii). Genetic predisposition.

The appearance of Heinz bodies is a feature of phenothiazine induced anaemia that has been observed in monkeys ¹³¹, horses ⁷⁶, dogs ¹³⁴ and mice ¹³¹. The administration of thionol to the latter rodent also produces this phenomenon ¹³¹. The fact that Heinz bodies can be demonstrated in the red cells following exposure to phenothiazine indicates that the cells are actually damaged. The degree of damage varies between individuals and may be partly attributed to an abnormality of the erythrocytes themselves.

Heinz bodies are usually present in increased numbers in the cells of patients with haemolytic anaemia that is caused by a congenital susceptibility to many drugs such as phenylhydrazine, naphthalene, the antimalarials, sulphonamides and other antibacterial agents ²⁶². This susceptibility is due to a relative deficiency in the activity of a specific enzyme, glucose-6-phosphate dehydrogenase (G6PD), the first enzyme of the hexose monophosphate shunt which catalyses the conversion of glucose-6-phosphate to 6-phosphoglucono- δ -lactone with the production of NADPH (Eq. 5).



A decrease in the activity of this enzyme leads to a decrease in the availability of NADPH and subsequent impairment of glutathione reduction, resulting under certain conditions in oxidative denaturation of haemoglobin and eventual lysis of the red blood cells.

This deficiency is inherited as an X-linked recessive disorder that has a high frequency amongst Negroes. In Africa about 20% of males (hemizygotes) and about 4% of females (homozygous for the abnormal gene) are affected. A small number of heterozygous females are also deficient of G6PD. A similar deficiency occurs in Caucasian and Mongoloid races where it is usually more severe. The disorder is not a clear cut presence or absence of activity but exists as many phenotypes possessing differing degrees of deficiency ²⁶².

Individuals with G6PD deficiency normally enjoy good health but are liable to haemolysis if any incriminated drug or food (eg. broad bean; Vicia faba - Favism) is ingested. The haemolytic effect is related to the dose and will not be clinically detectable if the amount does not exceed a critical level, which will vary with different individuals depending upon the degree of deficiency and other predisposing factors such as infection. Some doses are then non toxic. In certain types of G6PD deficiency the young erythrocytes do have some G6PD activity and remain viable until this activity decays when they become susceptible to haemolysis ²⁶².

Such an enzyme deficiency, which has explained the haemolytic anaemias caused in certain individuals by several offending drugs, may go part of the way to explain the haemolytic effects of phenothiazine. The inhibition of G6PD by various substituted phenothiazines has been demonstrated in vitro but phenothiazine and its sulfoxide were not examined owing to their low aqueous solubility ²⁶³. An interesting point is that the cases of severe haemolytic anaemia reported in the medical press appear to occur in young girls (6 to 10 years old) ^{130,153,174,175}, a reverse situation to that expected from the knowledge of the X-linked mode of inheritance of G6PD deficiency where it would be assumed that more males would show the deficiency (eg. haemophilia).

2). Neuromuscular problems.

Neuromuscular incoordination in cattle has been shown to be dose dependent; animals receiving 36 to 52 g (c. 440 mg/kg body wt.) of phenothiazine had no side effects whereas those receiving 250 g developed anorexia and incoordination of the hind legs ²⁶⁴. Again, variability amongst the same species is observed as well as that which exists between different species. It has been stated that of a thousand pigs treated with phenothiazine, only one mild reaction was encountered whereas, in other herds the majority of animals have reacted, some fatally ^{185,265}. It is quite possible that some local environmental factor is at play or perhaps a predisposing genetic susceptibility, exaggerated by herd inbreeding.

A rigid tetanic spasm with increased muscular tone, such as observed in pigs (opisthotonus) ¹⁸⁵, is usually associated with damage of the upper motor neurones as opposed to the localised flaccid paralysis seen with lower motor neurone lesions. The staggering gait with loss of balance and power of coordination ^{136,144,184-190} is suggestive of cerebellar involvement and possibly extrapyramidal lesions. Such problems are well known side effects of the N-substituted phenothiazine drugs such as chlorpromazine, prochlorperazine and promazine which have been reported as causing involuntary movement disorders and even seizures in some patients ²⁶⁶. It has been suggested that this effect is due to the interference of dopaminergic transmission in the basal ganglia ('extrapyramidal ganglia') owing to the spatial similarity of the phenothiazines and dopamine ²⁶⁷ (Fig. 7) and this could also be the case for phenothiazine itself which is known to be concentrated in the human brain ¹²³.

In addition to these central actions, peripheral effects on the neuromuscular junctions of skeletal muscle cells (nicotinic receptors) are also possible and must not be overlooked. Phenothiazine has been

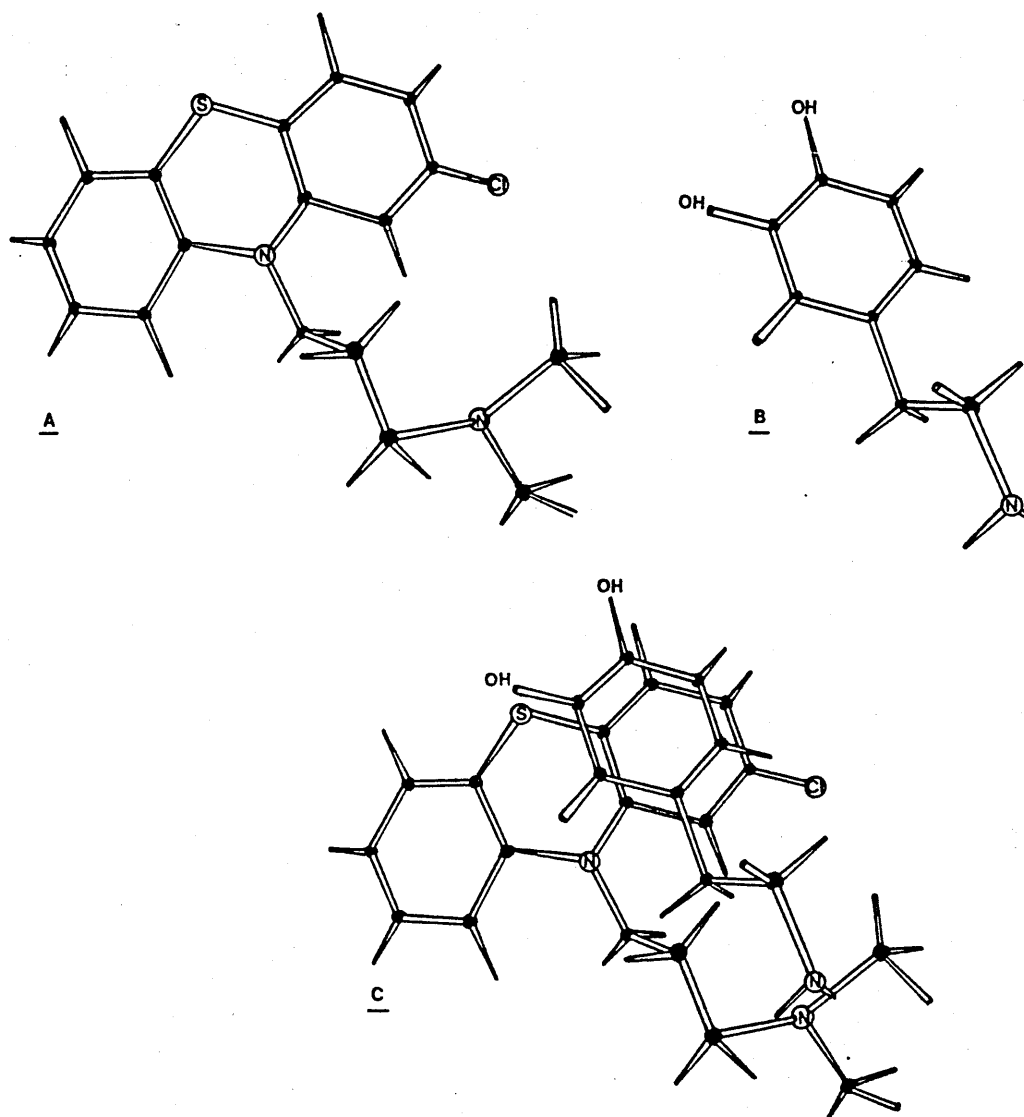


Fig. 7. Structural similarity of phenothiazine and dopamine.

Molecular models of chlorpromazine (A) and dopamine (B).
(C) Illustrates how dopamine may be superimposed on a portion of the chlorpromazine molecule (Ref. 267).

shown to depress neuromuscular transmission and raise the excitation threshold potential in the shore crab (Garcinus maenus) at very low concentrations (c. 0.5 μ M) ²⁶⁸ and this may be the result of a direct effect on the neuronal membrane. It is known that curare, an alkaloid which produces a non-depolarising blockade at cholinergic (nicotinic) terminals, has no effect at the nerve-muscle junction of the crab which are, therefore, different from the neuromuscular junctions found in mammalian systems where curare is a potent blocking agent ²⁶⁹.

Phenothiazine itself has no effect in vitro on liver flukes (Fasciola hepatica) and failed to evoke any response in the exposed neuromuscular apparatus of the roundworm (Ascaris sp.) ²⁷⁰⁻²⁷². Both the liver fluke and roundworm musculature are stimulated by acetylcholine and possess cholinergic components but it is not certain that the acetylcholine-cholinergic system is important in nematode physiology ^{236, 271, 273}. However, in vitro studies with phenothiazone, the oxidation product of phenothiazine, have shown it to produce paralysing effects on both the large roundworm (Ascaris lumbricoides) and the liver fluke, rapidly being fatal to the latter. Thionol and the sulfoxide were less effective but still produced paralysis in both parasites ^{274, 275}. Investigations in the horse have also shown phenothiazone to be a potent inhibitor of serum cholinesterase ^{245, 247} and if this inhibition also occurs with the enzyme from nervous tissue it may serve to explain these adverse reactions because of the role of acetylcholine in the mediation of nerve impulses at mammalian neuromuscular junctions ²⁶⁴. Again, despite much speculation, we have as yet no detailed overall explanation supported by adequate data of the mechanism of these ailments ²⁶⁴.

3). Photosensitization.

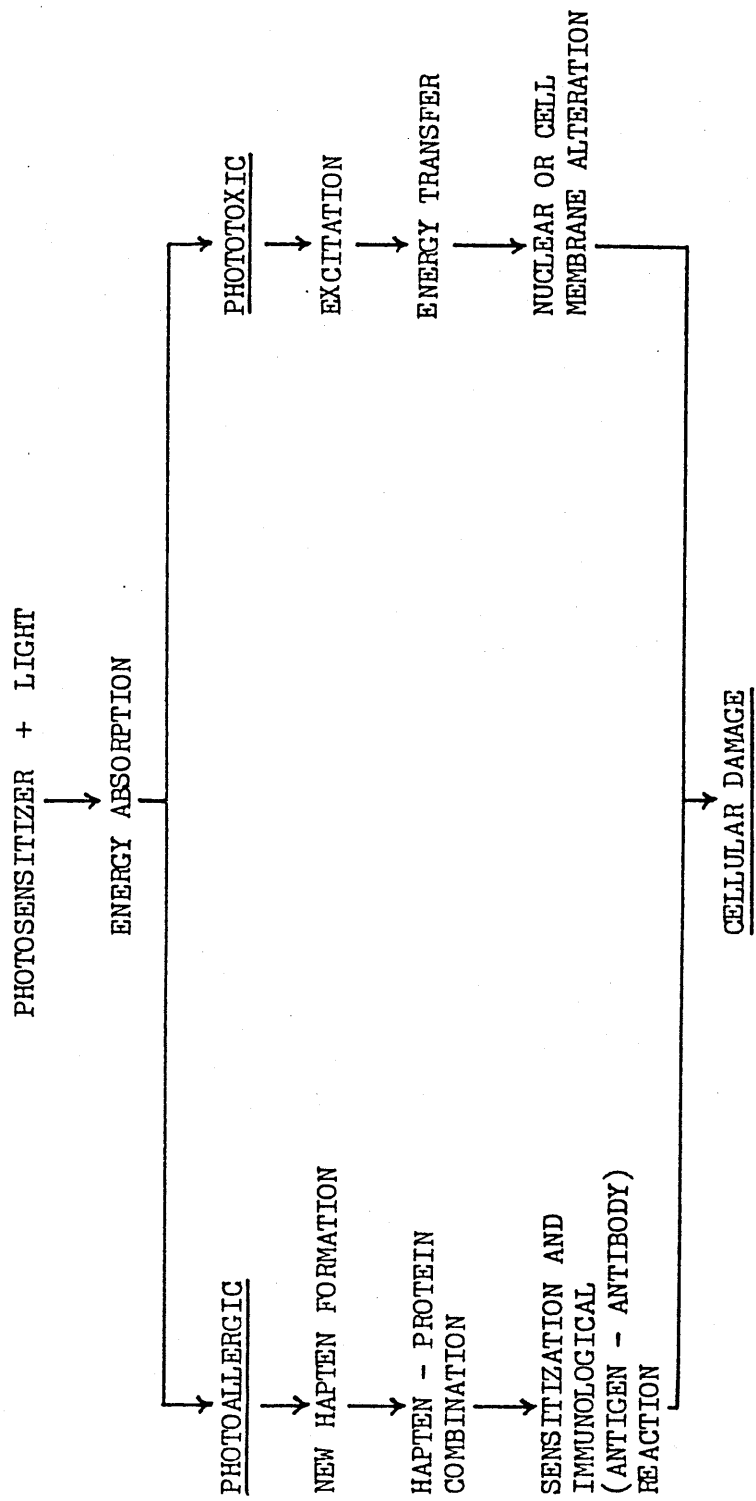
The basic mechanisms by which drug-induced photosensitivity reactions are mediated are outlined in scheme 4. Photosensitization may take the form of an exaggerated sunburn (phototoxicity) or may involve a delayed hypersensitivity reaction (photoallergy). The former process undoubtedly takes place with phenothiazine and the later mechanism probably plays a part in the overall picture.

(i). Phototoxicity.

The oral ingestion of phenothiazine or thionol and subsequent irradiation of exposed skin has been shown to give rise to a hyperaemic response ¹⁹⁵ and others have noticed a reaction resembling exaggerated sunburn after oral ingestion of relatively high doses of phenothiazine. One interesting report states that the exposure to infra-red radiation of horses, dogs and mice previously treated with phenothiazine resulted in rapid desquamation and the falling out of hair over the whole body surface. Severe skin pigmentation was also seen in dogs. Such effects were not produced by irradiation in the absence of ingested phenothiazine ²⁷⁶.

These reactions to systemic administration would seem to be phototoxic in nature and are probably traceable to a photosensitization product or products produced either directly or indirectly by the presence of the phenothiazone / leucophenothiazone or thionol / leuco-thionol redox systems present in the tissues resulting from the oxidation of absorbed phenothiazine ¹⁹⁵. Whilst the initial step of all photosensitivity reactions must be the absorption of light by the chemical or its metabolites, the precise mechanisms of photosensitivity are, for the most part, unknown ^{277,278}.

One reaction that has been adequately investigated for the causative agent is that of keratitis, inflammation of the cornea,



Scheme 4. Drug - induced photosensitivity.

which occurs in young cattle dosed with phenothiazine and subsequently exposed to sunlight. The injection of phenothiazone, leucophenothiazone sulphate, thionol or phenothiazine sulfoxide into the anterior chamber of the calf eye followed by a deliberate exposure to bright sunlight for 90 minutes resulted in the development of an intense keratitis some 20 hours later. The injection of other agents whose photosensitizing activity is well established (haematoporphyrin, phylloerythrin, hypericin, rose bengal) also produced intense keratitis under these conditions whereas control injections of saline did not.

Post mortem investigations of other calves dosed with phenothiazine showed that the only derivative present in the lacrimal fluid and aqueous humour of the eye was the sulfoxide; no sulfoxide being detected in the analogous parts of sheep who are not normally susceptible to this ailment except when given massive doses. The effective wavelength of light which produced this condition lay between 320 and 360 nm which corresponds to an absorption maximum in the sulfoxide spectrum, phenothiazine, phenothiazone and thionol lying outside this range (see Table 1 and 12) ^{14,55,63}. In addition, a sheep dosed with the sulfoxide was shown to subsequently develop keratitis ¹²². It was concluded that this particular ailment was a photosensitization phenomenon and that the photodynamic substance was phenothiazine sulfoxide.

The topical application of phenothiazine mixed with alcohol or wool fat (lanolin) to the forearm of volunteers was shown to produce no irritation even when exposed to irradiation and these investigators concluded that the undesirable effects reported in other workers were not due to a local mechanism but systemically mediated ¹⁹⁵. However, a case has been cited where an individual suffered photosensitization and sunburn-like irritation of the skin on parts of the body contaminated with phenothiazine dust, but no similar irritation on simultaneously

Table 12. Ultraviolet absorption maxima of phenothiazine and its derivatives.

Solvent	Compound			
	phenothiazine	phenothiazine sulphoxide	phenothiazine	thionol
methanol				
max (nm)	(log E)			
	318 (3.6)	336 (3.7)	508 (3.8)	592
	254 (4.5)	303 (3.9)	368 (4.0)	276
		271 (4.2)	272 (4.2)	232
		229 (4.5)	258	
			236 (4.2)	
acetonitrile				
max (nm)	(log E)			
	320 (3.7)	339 (3.7)	495 (3.9)	
	254 (4.7)	298 (3.9)	362 (4.0)	
		269 (4.1)	274 (4.2)	
			236 (4.3)	

exposed, but uncontaminated parts of the body ²⁶⁴. It has been stated that skin contact with freshly made phenothiazine will not induce a dermatitic reaction but once it has been allowed to oxidise then such adverse reactions are almost certain ²⁷⁹. The use of a solvent may enhance the entry of the compound into the lipid barrier of the skin. It is not yet clear which derivative of phenothiazine or whether or not all of the derivatives are the dermatitic agents although it is now fairly certain that topical application alone may induce a localised reaction in certain individuals. The additional complication that fine particulate phenothiazine may enter the body by inhalation whilst spraying must also be borne in mind.

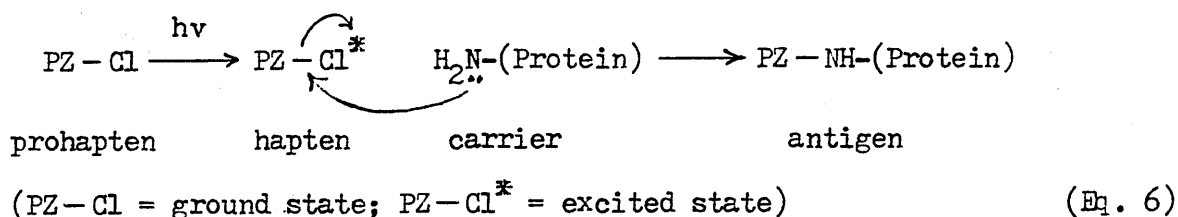
(ii). Photoallergy.

Acute dermatitis with exfoliation on the exposed areas of the the body has been described in patients who had worked on the tableting of phenothiazine, undoubtably under constant exposure, and it may be significant that virtually all of the cases where similar toxic reactions have occurred in man there has been repeated administration or contact with phenothiazine ²¹¹. This photocontact dermatitis probably has an immunological basis, similar to that due to chlorpromazine, and could be classified as photoallergic in nature.

The first stage in this procedure is the interaction of the drug with light to form a photoactivated compound; a hapten which can then combine with a protein or other macromolecule (carrier) to form the allergen possessing immunological properties (Scheme 4). The mechanism of the subsequent immunological response is, presumably, similar to other types of delayed hypersensitivity mediated through immunologically competent cells, principally lymphocytes ²⁸⁰.

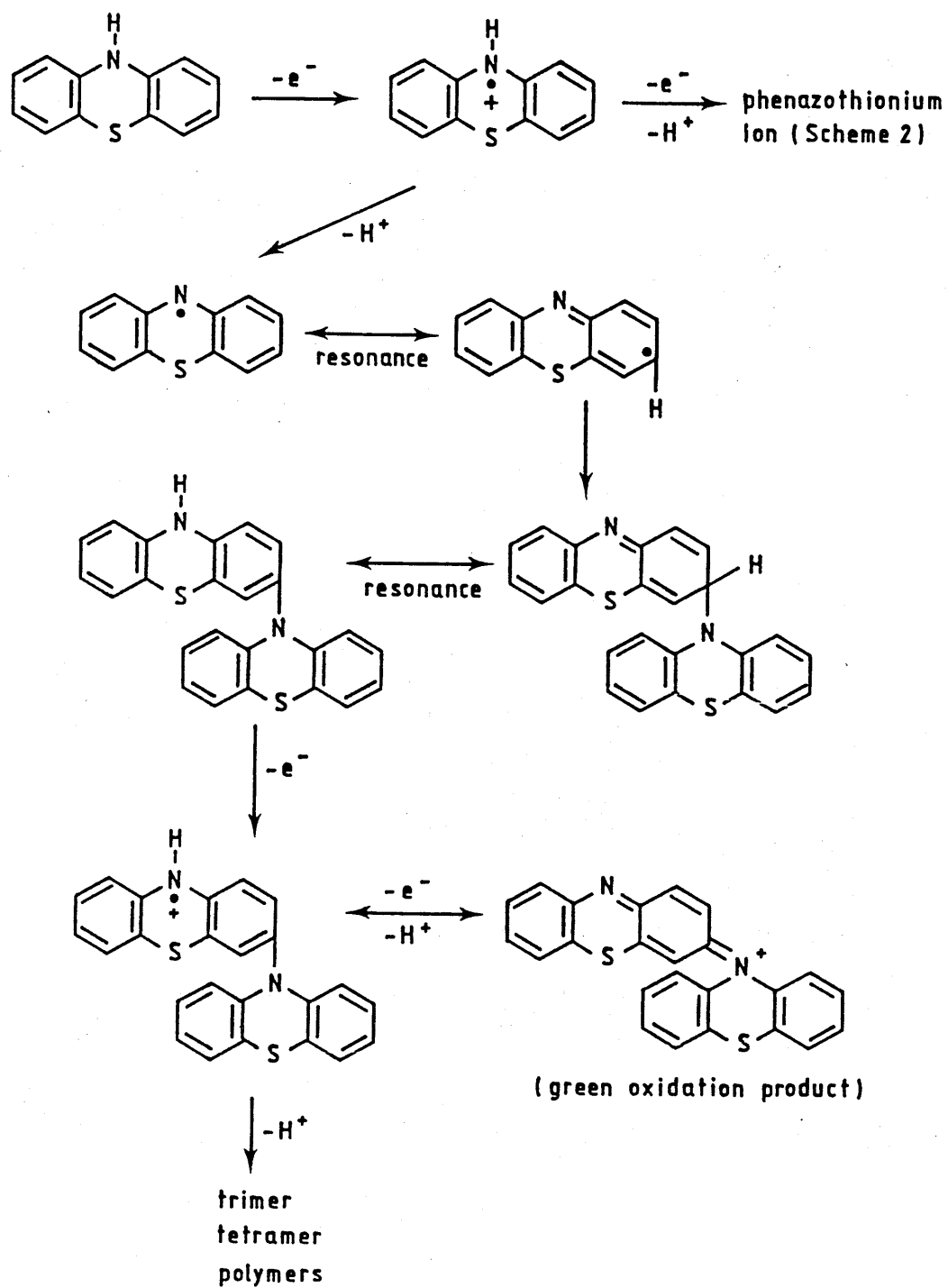
The identity of the reactive species in most instances is not known to any degree of certainty although there is much speculation.

For chlorpromazine and other 2-chlorophenothiazines (eg. perphenazine, prochlorperazine) the photoexcitability of the chlorine moiety has been implicated in which the activated molecule reacts with a free amino group on the carrier protein to form the antigen (Eq. 6). Without the chlorine moiety this particular reaction cannot occur ^{278, 281}.



However, it is possible that the parent molecule itself is capable of a photosensitizing effect. Other compounds possessing unsaturated tricyclic aromatic structures, usually containing nitrogen or oxygen atoms which have lone pairs of electrons which are not involved in actual bonding (eg. methylene blue, 2) have been shown to require only a small amount of photic energy to activate them. It has been suggested that phenothiazines, apparently through dimer products possibly acting as free radicals, produce the allergy and it has been shown that phenothiazine itself is capable of forming such dimeric and polymeric products (Scheme 5) ^{15,282,283}.

If this is truly the mechanism then a cross-sensitivity should be present within a sensitized individual to structurally similar molecules. This has been shown to be the case for promazine and chlorpromazine but such studies have not included the parent compound itself.



Scheme 5. Polymerization of phenothiazine. (Refs 14,15).

5. CONCLUDING REMARKS

For a compound that has enjoyed extensive and widespread use for more than two decades suprisingly little is known with any certainty about its mode of action. Its decline from popularity in the mid 1960's meant that many of the modern investigative techniques now readily accessible could not be applied to the study of this drug and the information that is available in the literature is usually incomplete or inconclusive when viewed under the light of modern scientific assessment.

What is apparent is that to be effective the compound is usually given in large doses and because of its high lipid solubility becomes widely distributed around the body. Here it undergoes enzymatically catalysed chemical alterations, mainly oxidations of the carbon and sulphur atoms and conjugation (condensation) reactions with glucuronic and sulphuric acids.

The insecticidal, antibacterial and anthelmintic actions of phenothiazine and the production of the unwanted toxic effects presumably arise from common underlying mechanisms; the latter perhaps manifest themselves in animals which may have an environmental or genetic predisposition to such reactions. Three basic ways in which the phenothiazine molecule interacts with the cellular components of living tissue to produce these observed effects have become apparent from evaluating the available literature.

1). Non-specific macromolecular disruption

- high lipid solubility permits disruption of the lipid phase of membranes.
- van der Waals interactions allow non-specific binding to the protein phase of membranes and to proteins including enzymes.

2). Redox systems

- permits energy transfer; disrupts cellular components, inhibits enzyme systems, photosensitization (phototoxic).

3). Allergen formation

- photoactivation; photosensitization (photoallergy), anaphylaxis.

It is probable that these three basic mechanisms explain most, if not all, of the actions of phenothiazine. However, much work still remains to be undertaken to uncover the elusive details and with phenothiazine now being the parent compound of a multitude of extensively used therapeutic agents perhaps the time is ripe for the compound to be reinvestigated using modern scientific techniques.

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